THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XXXV SEPTEMBER–OCTOBER, 1959 NUMBER 5

INJECTION-CORROSION STUDIES OF NORMAL AND CIRRHOTIC LIVERS*

MILTON R. HALES, M.D.†; JOHN S. ALLAN, M.D., and ERNEST M. HALL, M.D.

From the Department of Pathology of the University of Southern California School of Medicine and the Los Angeles County Hospital, Los Angeles, Calif.

Since the realization that portal hypertension was a consequence of vascular alterations within the cirrhotic liver, there have been numerous attempts to establish the nature and genesis of these vascular lesions, often with completely conflicting results. Almost a century ago, Frerichs[1] demonstrated by gelatin injections that in cirrhosis the hepatic venous capillaries became obliterated and the portal veins became narrowed and angular, while the hepatic arteries and the arterial bed were enlarged. He also described the development of new communications between all 3 vascular beds in the connective tissue septums. Over 60 years later, Kretz[2] noted that in gelatin injections and in corrosion specimens the hepatic veins were distorted and tortuous and had fewer small branches than normal. In the septums, the portal venous and hepatic arterial beds were increased, and there were new anastomoses between hepatic arteries and portal veins which, along with the obliteration of venous capillaries, contributed to the development of portal hypertension. Kretz also felt that because of the formation of similar new anastomoses between portal and hepatic veins, portal blood by-passed much of the parenchyma, and the latter was consequently supplied by hepatic arteries. A few years later, in 1907, Herrick[3] concluded from post-mortem perfusion studies in cirrhotic livers that there was an increased arterial inflow which, combined with an increased intercommunication between hepatic arteries and portal veins, was the most significant factor in the depression of portal venous flow and in the development of portal hyptertension.

*Supported by a grant from The Attending Staff Association of the Los Angeles County Hospital.

Presented in part at the Fifty-second Annual Meeting of the American Association of Pathologists and Bacteriologists, Houston, Texas, April 9, 1955.

Received for publication, December 29, 1958.

†Now at the Department of Pathology, Yale University School of Medicine, New Haven, Conn.

Segall,[4] in 1923, studied one cirrhotic liver in a series of radiopaque injections and found the hepatic arteries to be enlarged, with an extensive subcapsular arterial bed and numerous arterio-arterial anastomoses; he did not inject either venous bed.

In 1928, McIndoe[5] reported the results of perfusion and injection-corrosion studies which for the next two decades formed the basis of most thinking and writing on the subject. McIndoe concluded that in cirrhotic livers both venous beds were greatly distorted and reduced. As a consequence, most of the portal venous blood by-passed the liver through collaterals. Although the regenerated nodules were supplied by hepatic arteries, these vessels were not enlarged and eventually shared in the same atrophy as the veins; portal hypertension was attributed solely to venous obstruction. In 1942, Wakim and Mann[6] investigated the circulation in cirrhotic rat livers by a transillumination technique. They also noted that the blood supply of the regenerated parenchyma was arterial in nature, and as the cirrhotic process progressed, the venous and especially the arterial channels became tortuous. In the same year, Dock[7] repeated Herrick's simultaneous perfusion studies with essentially similar results. In some livers with alcoholic cirrhosis the arterial system was large and the arterial perfusibility increased. In all the examples of alcoholic cirrhosis there was increased communicability between hepatic arteries and portal veins. This, Dock thought, was a cause of portal hypertension in these cases. He suggested the possibility of lowering the high portal venous pressure by surgically reducing the arterial inflow, a procedure later to receive a disappointing clinical trial in the form of hepatic arterial ligation.

Moschcowitz,[8] in 1948, presented histologic evidence of anastomoses between portal and hepatic veins in cirrhotic livers, which he thought were newly formed vessels rather than persistent sinusoids. In 1950, Kelty, Baggenstoss and Butt[9] reconstructed a portion of a cirrhotic liver by the camera lucida technique and found that the site of vascular obstruction was on the hepatic venous side of the sinusoidal bed. The hepatic veins were displaced and flattened around regenerated pseudolobules as if by expansile pressure. Portal veins, protected by their natural sheath of connective tissue, were little affected by this process. Two years later, after they had developed a technique for estimating hepatic blood flow by application of the Fick principle to the clearance of bromsulphalein, Bradley, Ingelfinger and Bradley[10] were able to show that a significant reduction in total hepatic blood flow occurred in cirrhosis. This was the first direct physiologic evidence of this phe-

nomenon. By rapid serial angiography, however, Daniel, Prichard and Reynell[11] failed to find any impairment of speed or volume of flow of portal venous blood through cirrhotic rat livers, although sinusoidal filling was diminished and the venous trees were stunted and distorted.

In 1952, Popper, Elias and Petty[12] demonstrated by gelatin injections of cirrhotic livers that regenerated nodules were poorly supplied by portal venous blood. In the septums there were numerous anastomoses between portal and hepatic veins, and occasional arteriovenous anastomoses were present, mostly between hepatic arteries and portal veins. In corrosion casts of 4 cirrhotic livers, they described increased tortuosity, irregular spacing, and reduction in number of the vessels, as well as a basket-like arrangement about pseudolobules, and flattening of the hepatic veins. They postulated that the venovenous anastomoses, derived from sinusoids persisting in collapsed reticulum, diverted portal venous blood from the parenchyma and perpetuated the cirrhosis on an ischemic basis. Since the shunts were upstream from the sites of hepatic venous obstruction, they did not prevent the development of portal hypertension. The latter, incidentally, was ascribed to vascular obliteration and, possibly, transmission of arterial pressure into the venous network. The alterations subsequently noted by Mann, Wakim and Baggenstoss[13] in vinylite corrosion casts of cirrhotic livers were very similar to those described by Popper and his colleagues. No significant differences were seen in the cast of a liver with post-necrotic cirrhosis and one with alcoholic cirrhosis. Madden, Loré, Gerold and Ravid[14] reported more recently that in their neoprene casts of cirrhotic livers two patterns were observed. One, associated with chronic ascites, was characterized by an increase in the portal venous and hepatic arterial beds and a reduction in the hepatic venous bed. In the other, observed in cirrhosis without ascites or with ascites of the acute and reversible type, there was a symmetrical deficit in all of the vascular systems. In the former cases, these workers believed that the significant lesion was occlusion of hepatic veins by an obliterative fibrosis, whereas in the latter cases the hepatic venous obstruction was thought to be due to diffuse cellular edema.

From even this brief review of the more significant investigations of the vascular changes in cirrhotic livers, it is apparent that almost all conceivable alterations of the normal pattern have been reported at some time by one or more investigators: expansion of the hepatic arterial bed;[1-4,7,14] reduction in the arterial bed;[5] equal reduction in the two venous beds;[1,5,11] selective or more pronounced reduction in the hepatic venous bed;[2,9,12,14] anastomoses between portal veins and

hepatic veins,[1,2,8,12,13] between hepatic arteries and portal veins,[1-3,7,8,12] and between hepatic arteries and hepatic veins.[1,12] Venous obstruction was specifically attributed to the effect of fibrosis by some[1,5,11,14] and to expansile pressure of regenerated nodules by others.[9,12,13] Portal hypertension has been ascribed primarily to venous obstruction,[5,11,14] to transmission of arterial pressure through arteriovenous anastomoses,[3,7] and to a combination of these factors.[2,12]

It is also apparent from this historical survey that even when employing similar techniques in investigating the vascular alterations in cirrhosis, different workers have rarely made the same observations or been in entire agreement as to interpretations. Thus, even the morphologic end products of cirrhosis cannot be considered to be established until sufficient additional investigation has led to a common basis of understanding. As a contribution toward this end, the present paper reports the examination of a series of normal and cirrhotic livers by the injection-corrosion cast technique. Included in the investigation were 28 non-cirrhotic livers, 25 livers with portal cirrhosis, 6 with congestive (cardiac) cirrhosis, 3 with post-necrotic cirrhosis, and 3 with biliary cirrhosis.

Material and Methods

The technique of injection was modified from that described by Liebow, Hales, Lindskog and Bloomer[15] for preparation of bronchovascular corrosion casts of the lung. The injection masses were 8 to 12 per cent solutions of vinylite in acetone, usually with diatomaceous earth or lamp black added in amounts of 33 to 125 gm. per liter. Solutions were colored with appropriate acetone-soluble dyes. To insure uniformity in the injection masses used for different specimens, the viscosities were checked by viscometer just before injection. When indicated, the viscosity was adjusted by the addition of small amounts of concentrated vinylite solution or acetone.

The specimens were obtained from the necropsy service of the Los Angeles County Hospital. They were removed with the diaphragms still attached and with long segments of the inferior vena cava and the hepatoduodenal ligament. The inferior vena cava was cannulated just above the diaphragm and ligated below the liver. The portal vein, hepatic artery and common bile duct were cannulated in the hepatoduodenal ligament. While the liver was submerged in a water bath, a gentle stream of air was blown through each cannulated vessel to permit the identification and ligation of potential leaks.

In our initial studies, the vessels were first cleared of blood by perfusion with tap water and then acetone, but this procedure was later

abandoned when no beneficial effect could be demonstrated. The vinylite solutions were injected from aspirator bottles in which the desired pressure was maintained by means of a rubber hand bulb. The actual injection was completed and the plastic in small vessels partially solidified within a few minutes; blocks were then removed from the right and left lobes for subsequent histologic examination. To compensate for shrinkage of the injection masses as they solidified in the vessels, the cannulas were left attached to the aspirator bottles, under pressure, until the material in the largest vessels had become rigid (approximately 48 hours). The tissue was then digested in a large vat of concentrated hydrochloric acid, and the resultant cast was washed in running water for 24 hours, and when necessary, defatted in petroleum ether.

The masses used to fill the hepatic arterial system and the biliary ducts were made less viscid than those used in the two venous beds. In a few specimens, latex was used instead of vinylite to inject the arterial system and the biliary ducts. Although the relatively high viscosity of the vinylite solutions necessitated injection pressures 50 to 200 per cent higher than physiologic levels, extravasation was rarely encountered and was readily identifiable in the cast when it did occur. The order in which the different vessels were injected was varied from time to time, as were the injection pressures and the viscosities of the injection masses. With each of these variations in technique, normal livers were injected as standards of reference for any features observed in the abnormal livers. In each specimen, the hepatic veins and portal veins were injected at the same pressure with masses which were identical except for color.

Histologic sections were stained with hematoxylin and eosin and were graded by one of us (E.M.H.) from 0 to 4+ for the degree of fibrosis, fatty change, necrosis and regeneration. A histologic diagnosis as to the type of cirrhosis, if any, was also made. The clinical history of each patient was searched for evidence of alcoholism, dietary dyscrasia, previous hepatic or biliary tract disorder, ascites, varices or hematemesis. The necropsy protocols were reviewed for pertinent observations, including the presence of varices, ascites, splenomegaly or other possible signs of portal hypertension.

Results

Normal Livers

Since excellent detailed investigations of the vascular anatomy of normal livers are available,[16,17] only those features need be described here which pertain to the alterations occurring in cirrhosis.

The extent and pattern of the vascular injection were remarkably uniform in all livers considered to be normal grossly and microscopically. The injection masses penetrated equally far in the portal and hepatic venous systems, terminating in vessels between 100 and 200 μ in diameter with the masses most commonly used (Figs. 1 and 7). The small branches of the two venous beds interdigitated in such a manner that portal and hepatic veins were always removed as far as possible from each other. The pattern of division generally appeared to be dichotomous in type, although very small branches also entered or left the large parent trunks, often at very nearly right angles. The latter were particularly striking with fine injections of the hepatic veins, where tiny tributaries were often sufficiently numerous that the parent trunk had a bristly, fuzzy appearance. Portal veins appeared to be end-veins; only in the bed and wall of the gallbladder did separate branches intercommunicate. On the other hand, arcade-like subcapsular anastomoses between separate small hepatic veins were not at all uncommon, especially on the posterior surface and along the sharp inferior margin. No gross anastomoses between portal veins and hepatic veins were found in normal livers.

Within the liver there was one artery accompanying each portal vein, and situated between the portal vein and the bile duct; occasionally another much smaller artery was also present which communicated with the major artery. Through similar tiny vessels in the hilum there was often communication between the right and left hepatic arteries. The vinylite arterial injection mass terminated in vessels approximately 100 μ in diameter and hence did not fill vessels of the peribiliary plexus. No arteriovenous anastomoses were ever demonstrated.

In the wall and bed of the gallbladder, portal veins formed a network of anastomosing vessels, as did the arteries. The veins and arteries closely paralleled each other, the veins usually being paired and situated on each side of the artery. Although a small cystic vein often drained along the cystic duct into a large portal vein at the hilum, most of the veins drained toward the bed of the gallbladder and emptied through several trunks into portal veins within the hepatic parenchyma. There were never any tributaries of the hepatic veins in the wall of the gallbladder.

Injection of smaller radicles of the biliary ducts was unsuccessful, presumably because their viscid content could not be evacuated or forced back into canaliculi.

Portal Cirrhosis

Hepatic Arteries. In all of the casts of livers considered to represent portal cirrhosis grossly and histologically, there were gross alterations in the vessels. The most consistent variations from normal were enlargement of the hepatic arteries and increase in the injectable hepatic arterial bed (Figs. 2, 3 and 11). These features were observed to a minor degree even in livers with slight periportal fibrosis which could not be considered frankly cirrhotic. In severe cirrhosis, the arteries were not only enlarged but were often extremely tortuous. Instead of only one major arterial trunk accompanying each portal vein, there were usually 2 or 3 large intercommunicating arteries of nearly equal size in each portal zone.

As an indication of the magnitude of the hepatic arterial enlargement, the diameters of peripheral hepatic arteries were compared with the diameters of the adjacent portal veins. Several measurements were taken in random fields through the ocular micrometer of a dissecting microscope. Portal veins of 0.5 to 1.0 mm. in diameter were used as the standards of reference, and the relative diameters of the adjacent artery and vein were determined at this level. In normal livers the average diameter of these peripheral hepatic arteries was approximately one third that of the adjacent portal veins, whereas in cirrhosis the hepatic arteries averaged two thirds of the diameter of adjacent portal veins. In one cirrhotic liver the hepatic arteries were fully as large as the adjacent portal veins (Fig. 2). Such altered arteriovenous ratios in cirrhosis could, of course, result from a selective reduction in the diameter of portal veins rather than an increase in the diameter of hepatic arteries, but even casual inspection of the casts indicated that this was not the case. The injected portal venous bed often appeared almost as extensive as in non-cirrhotic livers, and the individual portal veins selected for measurement were not flattened and were rarely distorted. Considering also that 2 or 3 hepatic arteries of nearly equal size were often present with each portal vein, only one of which was measured, there was little question that in cirrhotic livers there was a striking increase in the total cross sectional area of the hepatic arteries.

Not only were hepatic arteries enlarged and their number in each portal zone increased, but the total injectable arterial bed was significantly expanded in cirrhotic livers (Figs. 3 and 11). This was manifested by the successful injection of the plastic into numerous *vasa vasorum* to the portal veins, small arteries enmeshing the bile ducts,

and small arteries ramifying within or beneath the capsule. In all of these sites the small arteries formed an anastomosing network. At the hilum, large communications between right and left main hepatic arteries made it possible to inject the bed of the left hepatic artery by way of the right hepatic artery, and vice versa. In one cirrhotic liver a peripheral hepatic artery communicated with a sizable diaphragmatic artery; the latter vessel was injected in retrograde manner. In the one liver examined in which the portal vein was occluded by an old thrombus, the hepatic arteries were enlarged and the arterial bed greatly expanded, but not beyond that seen in many cirrhotic livers with patent portal veins. The degree of hepatic arterial enlargement could not be shown to correlate significantly with the varying degrees of fibrosis or regeneration noted in the histologic sections, nor was it related to the weight of the liver.

With the injection mass most commonly used, the smallest arteries which were filled measured about 75 to 100 μ in diameter. In the cirrhotic as in the normal livers, no arteriovenous anastomoses were demonstrated. In a few livers, the arteries were injected with latex or with a low viscosity vinylite mass which penetrated some arteries of only 25 to 35 μ diameter. In several of the cirrhotic livers so injected, the arterial mass found its way into portal veins. Occasionally, normal livers similarly injected exhibited the same arteriovenous transfer, but to a much lesser degree. All attempts to identify the actual site of arteriovenous communication under the dissecting microscope were unsuccessful. In the histologic sections from these same livers, a few sinusoids which were dilated to 25 to 35 μ in diameter and contained the arterial injection mass were invariably found. It was thus impossible to state whether the arteriovenous communication was by way of parenchymal sinusoids or by way of vessels in the fibrous septums. At any rate, communication between hepatic arteries and portal veins seemed unduly free in cirrhotic livers, although it was apparently through vessels of minute size.

Another confirmation of the easy communication between hepatic arteries and portal veins in cirrhosis was incidentally encountered early in our studies when we were perfusing the vessels with tap water to remove the blood. As was noted by Herrick[3] in 1907, when the hepatic artery of a normal liver was perfused, the return of fluid was primarily by way of the hepatic vein, although there was also some return by way of the portal vein. In cirrhotic livers the reverse was usually true; the hepatic arterial perfusate was returned primarily, and sometimes exclusively, by way of the portal vein. Clamping the portal vein resulted in the development of extremely high

pressures, but still failed to increase significantly the meager return through the hepatic veins.

Portal and Hepatic Veins. In all of the vascular casts of livers with portal cirrhosis there was some reduction in the hepatic venous bed, and in 80 per cent of them there was also reduction in the portal venous bed (Figs. 4 and 8). Hepatic venous reduction was considered of severe degree in 60 per cent, whereas portal venous reduction was severe in only 36 per cent. Thus, in almost two thirds of the casts there was a disparity in the extent of the injection in the two venous beds, the hepatic being more severely reduced than the portal venous system. This disparity was particularly striking in 4 casts in which the portal venous bed appeared essentially normal but the hepatic venous bed was markedly restricted (Fig. 4).

Attempts at correlating the venous reduction independently with fibrosis or with the size of the pseudolobules was generally impossible because all but one of the livers with pseudolobules as large as 2 mm. also had severe fibrosis. However, in 8 of the 18 livers with severe fibrosis there were no pseudolobules over 2 mm. in diameter; 6 of these 8 livers had severe hepatic venous reduction, and 2 had severe portal venous reduction. This observation of severe venous reduction in the presence of only tiny, and usually widely separated pseudolobules suggests that fibrosis *per se*, independent of any expansile pressure from enlarging pseudolobules, had a role in the obstruction of small veins in the cirrhotic liver, and that this primarily involved the small hepatic veins. This selective effect on the small hepatic veins was well demonstrated in focal scars present in 4 specimens (Figs. 9 and 10). In these scars, histologic sections revealed only tiny, widely spaced nests of persistent hepatic parenchyma. In the casts, these zones were characterized by almost complete absence of hepatic veins, while the portal veins were not only well injected but actually appeared compacted and more numerous than normal (Figs. 5 and 6).

Although the close association of severe fibrosis and large pseudolobules made it impossible to demonstrate that vascular obliteration could be produced solely by expanding pseudolobules, there was no question of the relationship of the pseudolobules to venous distortion. All of the 12 livers with pseudolobules measuring 3 mm. or more had significant hepatic venous distortion, and in 66 per cent of these it was of severe degree. Nine of the 12 livers had significant portal venous distortion; this was severe in 4 cases. Significant distortion was rarely seen in the absence of large pseudolobules, regardless of the severity of the fibrosis. Venous distortion, like venous reduction,

was nearly always more severe in the hepatic veins than the portal veins; in only 2 livers was the distortion of portal veins considered equal to or greater than that of the hepatic veins. Under the dissecting microscope, the small distorted hepatic veins often appeared to have been abruptly flattened and displaced to the side, as if by pressure of a small expanding mass of tissue (Fig. 12). There was striking similarity to the appearance of the hepatic veins in the camera lucida reconstruction of Kelty and co-workers.[9]

A marked degree of fatty alteration was present in only 5 of the 25 livers with portal cirrhosis; 6 others had a slight amount, and the other 14 had minimal or no fat. That fat was not essential to venous reduction was indicated by the fact that 8 of the livers with severe hepatic venous reduction had minimal or no fat. Fatty change may contribute to vascular obstruction, however, as Himsworth[18] has contended. The two livers in which the degree of hepatic venous reduction seemed excessive for the degree of fibrosis or nodularity were severely fatty.

The weight of the livers correlated with the degree of fibrosis, the size of the nodules, and the severity of the venous reduction. Of the 8 livers weighing less than 1,400 gm., all had marked fibrosis, 7 had nodules larger than 2 mm., 7 had marked hepatic venous reduction, and 5 had marked portal venous reduction. Of the 12 livers with cirrhosis, weighing over 2,000 gm., only 6 had marked fibrosis, 5 had nodules larger than 2 mm., 4 had severe hepatic venous reduction, and only 1 had severe portal venous reduction.

In the cases of portal cirrhosis there did not appear to be any correlation between the disparity in venous reduction and the presence of chronic ascites, as reported by Madden and his colleagues.[14] Half of the patients with a disparity in the extent of the injection in the two venous beds did have evidence or history of chronic ascites, but the other half either had no ascites or developed ascites terminally for the first time.

Venovenous Anastomoses. Since the masses injected into the venous beds were relatively viscid, the casts indicated the presence or absence of only sizable communications between separate veins. As a consequence of the disproportionate reduction in the hepatic venous bed in cirrhosis, gross anastomoses (0.1 to 1.0 mm.) were present between separate hepatic veins in only 28 per cent of the cirrhotic livers compared with 46 per cent of the non-cirrhotic specimens. Anastomoses between separate portal veins were present in 4 (16 per cent) of the cirrhotic livers, but were present in only 2 (7 per cent) of the non-cirrhotic livers; both of the latter were small and atrophic, although

otherwise unremarkable. Gross anastomoses between portal veins and hepatic veins were present in only 2 (8 per cent) of the cirrhotic livers, one of these being an atypical portal cirrhosis in a patient with severe, prolonged and untreated hyperthyroidism. Such portal-hepatic vein anastomoses were also present in 2 non-cirrhotic livers with severe passive congestion, and in focal transverse bands of subcapsular atrophy and fibrosis in 2 otherwise unremarkable livers. In one of the latter and in the case of "thyrotoxic cirrhosis," some of the anastomoses measured over 1 mm. in diameter (Fig. 15). Remarkable dilatation of the venous trunks supplying and draining the anastomoses indicated an appreciable shunt of blood through them during life.

Intrahepatic Collaterals. In the casts of cirrhotic livers, there was consistent enlargement of certain veins communicating between intrahepatic branches of the portal vein and branches of the systemic venous system. Although measurements indicated no consistent dilatation of the main portal vein, the small veins which normally run in the *porta hepatis* and communicate with portal veins within the liver were often appreciably dilated. In most examples of cirrhosis there was a venous trunk extending from the left portal vein into the falciform ligament in the normal course of the umbilical vein. Although a tiny similar vessel was occasionally injected in a non-cirrhotic liver, in cirrhosis it was usually present and was of appreciable size. Indeed, in 4 casts such vessels measured over 5 mm. in diameter (Figs. 11 and 13). In only 1 of these 4 cases was a diagnosis of Cruveilhier-Baumgarten syndrome made clinically.

In addition to these vessels representing patent and dilated umbilical veins, there was often an entirely separate group of collateral vessels entering the falciform ligament. These were usually multiple, and while occasionally arising from the left portal vein near the normal entrance of the umbilical vein, they more often took origin from distal portal veins at the anterior margin of the liver (Fig. 14). Portal veins there failed to diminish in caliber as the capsule was approached and continued into the falciform ligament after a short tortuous course near or in the capsule. These vessels were often of appreciable size, measuring in 2 cases over 5 mm. in diameter. In normal livers there were often tiny extensions into the falciform ligament from adjacent peripheral portal veins, and it was presumably from these that the large para-umbilical collaterals developed in cirrhosis. Because of their empty, collapsed state, such collaterals were rarely recognized at necropsy unless specifically sought.

Diaphragmatic veins were often injected from the inferior vena cava, along with the hepatic veins. They sometimes seemed unusually

numerous and dilated, but they never established gross communications with either portal or hepatic veins in portal cirrhosis. In only one example of portal cirrhosis were communications demonstrated between intrahepatic branches of the hepatic arteries and arteries outside the liver; a very fine arterial injection disclosed communications between capsular branches of the hepatic artery and diaphragmatic arteries, the latter being injected in retrograde fashion. None of the cases investigated had had surgical ligation of the hepatic artery.

Post-necrotic Cirrhosis

Although none of the livers examined had the classical gross appearance of the "lobar" type of post-necrotic cirrhosis, 3 cases had microscopic features and clinical histories suggestive of a post-necrotic origin and were so classified. In another 3 cases, alcoholism and dietary dyscrasia were specifically and reliably denied by the patients, but since in these 3 the livers were grossly and histologically indistinguishable from portal cirrhosis, they were included in the latter group. Whether or not this was justified is of little importance in the present study since the casts of all 6 differed in no significant manner from the other instances of portal cirrhosis in patients with a strong history of alcoholism or dietary dyscrasia.

Congestive Cirrhosis

Six livers studied were considered to have the microscopic features of congestive or cardiac cirrhosis. In 5, central and portal fibrosis was minor, there was minimal lobular distortion, and the vascular pattern in the casts differed from that of normal livers only in dilatation of the hepatic veins and slight arterial enlargement. One liver, however, from a patient with tricuspid insufficiency, had severe cirrhosis with significant fibrosis and even pseudolobule formation. In this case, hepatic arteries were large, the hepatic veins were remarkably dilated, and the hepatic venous bed was more extensively filled than the portal venous bed (Fig. 16). On the diaphragmatic surface communications were present between hepatic veins and diaphragmatic veins, and between enlarged hepatic arteries and diaphragmatic arteries. A striking lesion in this instance consisted of large para-umbilical collateral branches of the portal veins entering the falciform ligament at the sharp anterior margin of the left lobe. Portal hypertension in this case was not solely a reflection of high systemic venous pressure, but was sufficiently augmented by intrahepatic venous obstruction that collateral pathways had developed to by-pass this obstruction.

Biliary Cirrhosis

One of the 3 cases of biliary cirrhosis had extreme focal dilatation of biliary ducts. In these sites the hepatic arteries were enlarged, the hepatic veins completely absent, and the portal veins markedly distorted and reduced in size and number. The 2 other specimens, from patients with portal hypertension, also manifested hepatic arterial enlargement but were especially remarkable for the fact that the portal venous reduction was more severe than that of the hepatic venous bed. Many portal veins were abruptly obliterated in a segmental fashion as if by thrombosis, and their distal segments were injected in retrograde manner from collateral communications with adjacent unobstructed portal veins. This observation correlated well with the presence of thrombophlebitis of small portal veins observed microscopically in these livers. MacMahon[19] has commented on the frequency of thrombophlebitis of small portal veins complicating cholangitis. Incidentally, similar obstructions of portal veins, with distal collateral communications, were observed in the walls of large congenital cysts present in two otherwise normal livers.

Discussion

The vascular alterations in the casts of livers with portal cirrhosis support the concept that the initial venous obstruction in this disorder is in the small hepatic veins rather than in the portal veins. In a majority of such casts, hepatic venous reduction was more severe than portal venous reduction, and in some, the portal venous bed was little if at all altered. The observation that large pseudolobules were invariably associated with distortion and often with flattening of the hepatic veins indicates that the expansile pressure of regenerating nodules may well be a factor in the venous obstruction, as originally proposed by Kelty and his associates.[9] However, there was an impressive independent relationship between fibrosis *per se* and reduction of the hepatic venous bed in our casts of cirrhotic livers. This was especially apparent in broad scars devoid of pseudolobules, where the hepatic venous bed was restricted invariably and profoundly.

How fibrosis results in obliteration of the small veins, and especially the small hepatic veins, is not known. The force exerted by the contraction of maturing collagenous fibers may be the responsible agent, just as it is responsible elsewhere for the vascular obliteration in granulation tissue as the latter becomes converted into avascular scar. Whatever the mechanism of venous obstruction, whether by expansile pressure of regenerating nodules or by contraction of septal connective

tissue, the hepatic veins are much more susceptible than the portal veins. The latter may be afforded some protection by their natural sheath of periportal connective tissue.

We could not escape the impression that as the cirrhotic process became more severe, the degree of portal obstruction tended to approach or equal the degree of hepatic venous obstruction, and that this was the reason for the apparently equal venous reduction noted in some cases. Other examples of equal reduction of the two venous beds, however, appeared to be instances of relatively early cirrhosis and must be explained on another basis. As Phillips and Davidson[20] have noted, individuals dying of early cirrhosis often have severely fatty livers and acute parenchymal necrosis. In our casts of early cirrhosis it is likely that the observed venous reduction was a reflection of sinusoidal obliteration by the swollen, fatty and necrotic parenchymal cells. For a successful vascular injection, it is essential that there be a reservoir of sinusoids or capillaries to receive the blood and air displaced from larger vessels by the injection mass. In livers with early, fatty cirrhosis this sinusoidal reservoir must be appreciably reduced.

Our coarse injections did not fill vessels of the size of the anastomoses which Popper, Elias and Petty[12] found so numerous in their delicate gelatin injections of cirrhotic livers. We did, however, demonstrate gross anastomoses of this type in two of the cirrhotic livers. Surprisingly, we also encountered them in two livers with severe chronic passive congestion and in two others with only focal zones of subcapsular atrophy and fibrosis. These observations are in perfect accord with the conclusion of Popper and his colleagues that such venovenous anastomoses represent dilated sinusoids persisting in collapsed reticulum. Although our casts demonstrate that these anastomoses may on occasion attain macroscopic size, in most cirrhotic livers they must either be very small, or infrequent, or, as Popper suggests, "upstream" from the site of hepatic venous obstruction; otherwise, cirrhosis would not be complicated by portal hypertension.

The enlargement of hepatic arteries, with expansion of the arterial bed, is in conformity with the observations of both the older and more recent investigators. Dock[7] and Madden and co-workers[14] have made similar observations, although inconstantly. On a theoretical basis, such enlargement should be expected, for there is evidence in other studies that regenerated pseudolobules are supplied primarily by arterial blood.[2,3,6] The connective tissue of the fibrous septums must also be supplied by hepatic arteries, since arterial enlargement is almost as conspicuous in broad scars essentially devoid of paren-

chyma as in regions of closely grouped pseudolobules. Indeed, arterial enlargement is even evident in livers with portal fibrosis but without lobular distortion or true cirrhosis. Increased intercommunication between hepatic arteries and portal veins has been described in several reports, and Popper and his associates[12] claim to have demonstrated true arteriovenous fistulas anatomically by gelatin injection. In other organs, arteries supplying even tiny arteriovenous fistulas are enlarged as a reflection of their increased flow. This might well be another factor contributing to hepatic arterial enlargement in cirrhotic livers. A final explanation for the arterial enlargement concerns the entire adjustment of parenchymal circulation that must occur in sites where and when small hepatic veins are selectively obliterated. Circulation there must utilize the only available venous bed, that of the portal veins, for efferent flow, and hence must rely solely on hepatic arteries for the afferent supply to the parenchymal sinusoids. Thus, in regions where hepatic veins are obstructed, arterial blood, after traversing the parenchymal sinusoids, must flow in retrograde manner in portal veins. It may then be shunted into extrahepatic collaterals of the falciform ligament, into anastomoses between portal veins and hepatic veins, or into sinusoids in regions where hepatic veins still persist and drain parenchyma.

The blood supply of the parenchyma in sites of hepatic venous obliteration must resemble that of the gallbladder, being supplied solely by the hepatic arteries and drained solely by portal veins. The possibility of localized retrograde flow has been suggested by others.[21-23] In casts with large regions essentially devoid of small hepatic veins, yet with a fairly well preserved portal venous bed and an expanded arterial bed, the conclusion is inescapable that the portal veins must be functioning as efferent channels for the blood supplied to the parenchyma and the connective tissue by the hepatic arteries. It is doubtful if such retrograde flow in the portal veins often involves the entire liver or extends to the main portal vein. There is, however, at least one case on record in which the latter phenomenon was noted at operation for the surgical treatment of portal hypertension.[21]

The hepatic arterial enlargement and the expansion of the arterial bed in cirrhosis indicate that the ratio of hepatic arterial to portal venous blood flow in the cirrhotic liver must be appreciably higher than the 1:3 figure generally accepted for the normal liver, but how much so is unknown and must vary appreciably from case to case. From the change in estimated hepatic blood flow following portocaval shunting procedures, Bradley, Smythe, Fitzpatrick and Blakemore[24] concluded that approximately 75 per cent of the blood flow to the

cirrhotic liver was from the hepatic artery. This was the average figure, and the range was extremely broad. Myers[25] arrived at a similar figure in one of the cases he studied by a sulfathiazole dilution technique.

McIndoe[5] estimated from his perfusion studies that at least 87 per cent of the portal venous blood by-passed the cirrhotic liver through portocaval collaterals. This figure, combined with more recent evidence that total hepatic blood flow in cirrhotic patients is about two thirds normal,[10] would also indicate that the major blood supply to the cirrhotic liver is from the hepatic arteries. Although these and other observations[3,7] support the morphologic evidence of an increased arterial blood supply to the cirrhotic liver, actual determination of the degree of arterialization must await the development of a reliable technique for measuring relative arterial and portal venous blood flows in normal patients, those with cirrhosis, and experimental animals.

If the hypothesis is correct that because of the obliteration of small hepatic veins the cirrhotic liver primarily becomes an arterial organ, then the treatment of portal hypertension by hepatic arterial ligation is obviously an entirely nonphysiologic procedure, saved from higher morbidity and mortality by the remarkable ability of the hepatic arteries to develop new collateral supplies. Similarly, any attempt to increase hepatic blood flow in cirrhosis by arterializing the portal vein, as is being presently contemplated by some,[26-30] may be destined to equally costly failure. Such a procedure may increase the circulation to those regions where parenchymal sinusoids are still drained by hepatic veins. But in sites where hepatic veins have been obliterated and an active parenchymal circulation is maintained only by means of retrograde flow in portal veins, arterialization of the portal vein must decrease rather than increase the supply of blood to the hepatic cells. Should such regions of arterial-parenchymal-portal circulation be extensive, arterialization of the portal vein might be disastrous, decreasing rather than increasing total hepatic blood flow. Indeed, it seems likely that in the normal course of cirrhosis it is the elimination of this retrograde portal venous flow by obliteration of the small portal veins that is responsible for the increasing circulatory impairment and functional insufficiency in the final stages of the disease.

Summary

An investigation of injection-corrosion casts of a large series of normal and cirrhotic livers revealed consistent enlargement of the hepatic arteries and arterial bed in cirrhosis, with an increased inter-

communication between hepatic arteries and portal veins. The venous beds were reduced and often distorted, these effects being most pronounced in the hepatic veins. Venous reduction was often associated with fibrosis, in the absence of any possible compressive effect from expanding pseudolobules. Occasionally, gross anastomoses were demonstrated between portal veins and hepatic veins in cirrhotic livers; they were also seen in some livers with severe chronic passive congestion, and in livers with focal bands of subcapsular atrophy and fibrosis. Umbilical and para-umbilical collaterals were surprisingly large and frequent in portal cirrhosis.

No significant differences were noted in the vascular pattern of portal and post-necrotic cirrhosis. In biliary cirrhosis, portal venous obstruction was often more severe than hepatic venous obstruction, presumably because of portal thrombophlebitis secondary to recurrent cholangitis. Congestive or cardiac cirrhosis was associated with dilatation of large hepatic veins, and, in one case with severe cirrhosis, with large para-umbilical collateral veins.

The possible genesis and physiologic effects of the vascular alterations in portal cirrhosis are discussed, including the probability of local retrograde flow of blood in the portal veins in sites where the parenchymal circulation is purely arterial in nature. It is suggested that these observations contraindicate surgical ligation of the hepatic artery or arterialization of the portal vein in the treatment of portal hypertension due to cirrhosis.

REFERENCES

1. Frerichs, F. T. A Clinical Treatise on Diseases of the Liver. Murchison, C. (trans.). The New Sydenham Society, London, 1861, Vol. 2, pp. 28–30.
2. Kretz, R. Lebercirrhose. *Verhandl. deutsch. path. Gesselsch.*, 1904, **8**, 54–59.
3. Herrick, F. C. An experimental study into the cause of the increased portal pressure in portal cirrhosis. *J. Exper. Med.*, 1907, **9**, 93–104.
4. Segall, H. N. An experimental anatomical investigation of the blood and bile channels of the liver; with special reference to the compensatory arterial circulation of the liver in its relation to surgical ligation of the hepatic artery—report of a case of arteriosclerotic aneurysm of the gastroduodenal artery. *Surg. Gynec. & Obst.*, 1923, **37**, 152–178.
5. McIndoe, A. H. Vascular lesions of portal cirrhosis. *Arch. Path.*, 1928, **5**, 23–42.
6. Wakim, K. G., and Mann, F. C. Effect of experimental cirrhosis on the intrahepatic circulation of blood in the intact animal. *Arch. Path.*, 1942, **33**, 198–203.
7. Dock, W. The rôle of increased hepatic arterial flow in the portal hypertension of cirrhosis. *Tr. A. Am. Physicians*, 1942, **57**, 302–306.

8. Moschcowitz, E. Laennec cirrhosis; its histogenesis with special reference to the role of angiogenesis. *Arch. Path.*, 1948, **45**, 187–215.

9. Kelty, R. H.; Baggenstoss, A. H., and Butt, H. R. The relation of the regenerated liver nodule to the vascular bed in cirrhosis. *Gastroenterology*, 1950, **15**, 285–295.

10. Bradley, S. E.; Ingelfinger, F. J., and Bradley, G. P. Hepatic circulation in cirrhosis of the liver. *Circulation*, 1952, **5**, 419–429.

11. Daniel, P. M.; Prichard, M. M. L., and Reynell, P. C. The portal circulation in experimental cirrhosis of the liver. *J. Path. & Bact.*, 1952, **64**, 53–60.

12. Popper, H.; Elias, H., and Petty, D. E. Vascular pattern of the cirrhotic liver. *Am. J. Clin. Path.*, 1952, **22**, 717–729.

13. Mann, J. D.; Wakim, K. G., and Baggenstoss, A. H. Alterations in the vasculature of the diseased liver. *Gastroenterology*, 1953, **25**, 540–546.

14. Madden, J. L.; Loré, J. M., Jr., Gerold, F. P., and Ravid, J. M. The pathogenesis of ascites and a consideration of its treatment. *Surg. Gynec. & Obst.*, 1954, **99**, 385–391.

15. Liebow, A. A.; Hales, M. R.; Lindskog, G. E., and Bloomer, W. E. Plastic demonstrations of pulmonary pathology. *J. Technical Methods*, 1947, **27**, 116–129.

16. Elias, H., and Petty, D. Gross anatomy of the blood vessels and ducts within the human liver. *Am. J. Anat.*, 1952, **90**, 59–111.

17. Andrews, W. H. H.; Maegraith, B. G., and Wenyon, C. E. M. Studies on the liver circulation. II. The micro-anatomy of the hepatic circulation. *Ann. Trop. Med.*, 1949, **43**, 229–237.

18. Himsworth, H. P. Lectures on the Liver and Its Diseases. Blackwell Scientific Publications, Oxford, 1947, ed. 1, 204 pp.

19. MacMahon, H. E. Biliary cirrhosis. Differential features of the five types. *Lab. Invest.*, 1955, **4**, 243–261.

20. Phillips, G. B., and Davidson, C. S. Acute hepatic insufficiency of the chronic alcoholic. Clinical and pathological study. *A. M. A. Arch. Int. Med.*, 1954, **94**, 585–603.

21. Egbert, H. L., and Raber, R. M. The physiologic effect of hepatic artery and portal vein ligation: results of perfusion studies and observations on patients at operation. *Surgical Forum*, 1951, **2**, 153–158.

22. Taylor, F. W., and Rosenbaum, D. The case against hepatic artery ligation in portal hypertension. *J. A. M. A.*, 1953, **151**, 1066–1069.

23. Popper, H., and Schaffner, F. Liver: Structure and Function. The Blakiston Div., McGraw-Hill Book Co., 1957, p. 284.

24. Bradley, S. E.; Smythe, C. M.; Fitzpatrick, H. F., and Blakemore, A. H. The effect of a portacaval shunt on estimated hepatic blood flow and oxygen uptake in cirrhosis. *J. Clin. Invest.*, 1953, **32**, 526–537.

25. Myers, J. D. The hepatic blood flow in Laennec's cirrhosis, with an estimate of the relative contributions from the portal vein and hepatic artery. *J. Clin. Invest.*, 1950, **29**, 836–837.

26. Schilling, J. A.; McKee, F. W., and Wilt, W. Experimental hepatic-portal arteriovenous anastomoses. *Surg. Gynec. & Obst.*, 1950, **90**, 473–480.

27. Cohn, R., and Herrod, C. Some effects upon the liver of complete arterialization of its blood supply. *Surgery*, 1952, **32**, 214–218.

28. Fisher, B.; Russ, C.; Fedor, E.; Wilde, R.; Engstrom, P., and Fisher, E. R. Further experimental observations on animals with arterialized livers. *Surgery*, 1955, 38, 181–193.

29. Jones, S. A.; Reynolds, T. B.; Schultz, E. B., and Gregory, G. Arterialization of the human liver following portocaval anastomosis; case report. *West. J. Surg.*, 1955, 63, 574–579.

30. Servello, M., and Petronio, R. Some experimental methods for arterialization of the liver: the aortosplenic anastomosis. *J. Internat. Coll. Surgeons*, 1956, 25, 448–454.

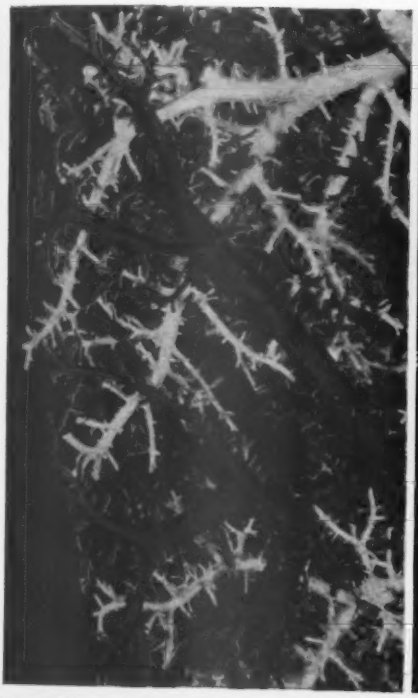
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Portion of the cast of a normal liver. There is regular interdigitation of the distal portal (red) and hepatic veins (white). Even sizable hepatic veins receive numerous tiny tributaries at almost 90° angles. The distal hepatic arteries (black) are rarely more than one third the diameter of the portal veins they accompany. Small biliary ducts were not injected. × 2.4.

FIG. 2. Portion of the cast of a cirrhotic liver. Hepatic arteries (yellow) are increased in number and are often fully as large as the adjacent portal veins (red). At the right margin some of the small portal veins are well injected, whereas only the larger hepatic veins (blue) persist. × 1.4.

FIG. 3. Cast of a liver with portal cirrhosis. In the expanded arterial bed the enlarged hepatic arteries (yellow) almost completely hide the stunted portal veins (red) and the even more severely stunted hepatic veins (blue). × 0.6.



1

2

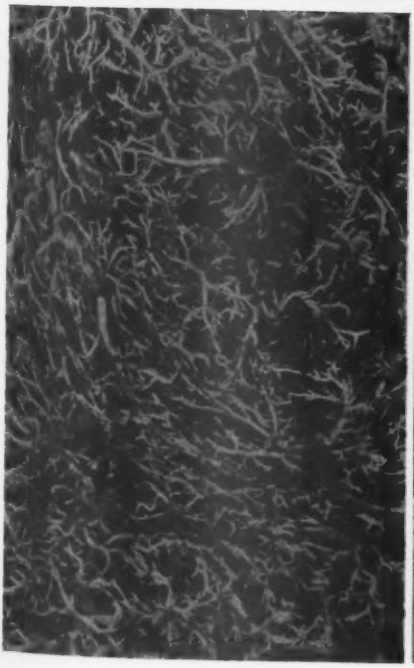
3

FIG. 4. Cast of a liver with portal cirrhosis. The portal venous bed (red) is little if at all altered from the normal, whereas the hepatic venous bed (white) is markedly restricted. This pattern of disproportionate reduction in the two venous beds was noted in two thirds of the livers with portal cirrhosis. The hepatic arterial bed (black) is greatly expanded. × 0.5.

FIG. 5. Portion of the cast of the cirrhotic liver shown in Figure 9. Distorted portal veins (white) are compacted in a broad depressed scar. Hepatic veins (blue) are essentially absent in the scar, but are present and severely distorted in the large bulging nodule of regenerated parenchyma seen at the right center. Hepatic arteries (red) are enlarged. × 1.5.

FIG. 6. Posterior surface of the right half of the cast of the cirrhotic liver shown in Figure 10. In the depressed triangular scar at the right margin, portal veins (white) are compacted and appear even more numerous than normal, but few hepatic veins (blue) could be injected there. Hepatic arteries (red) are enlarged, even in the scar. × 0.5.

4

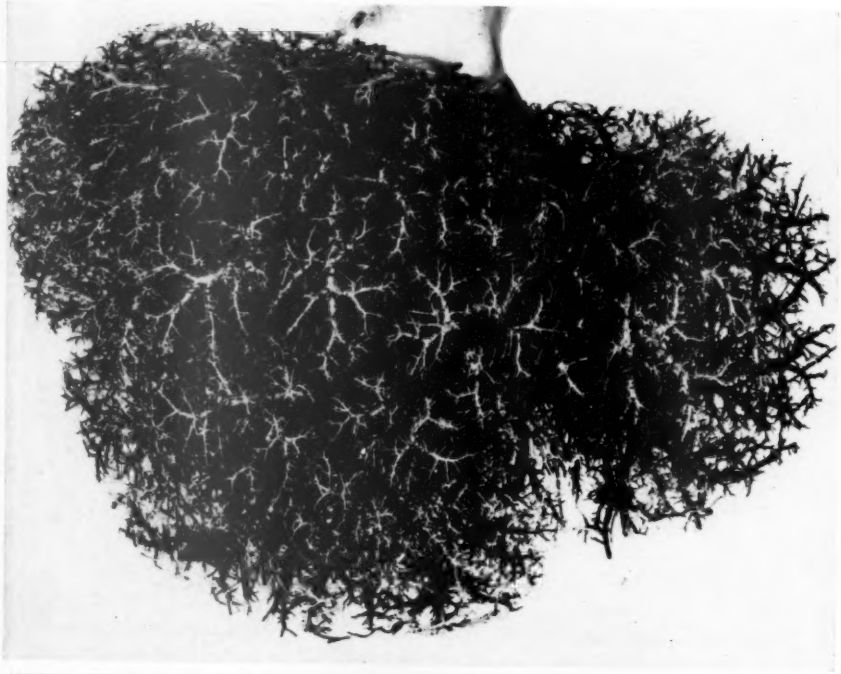


5

6

FIG. 7. Cast of a normal liver. Portal veins (gray) and hepatic veins (white) have been injected equally far to the periphery where their distal branches alternate uniformly. Hepatic arteries (black) are too small to be seen well at this magnification. × 0.55.

FIG. 8. Cast of a liver with portal cirrhosis. In this specimen the portal (dark gray) and the hepatic venous beds (light gray) are equally reduced, a pattern noted in only one third of the cirrhotic livers studied. Only the right hepatic artery (black) was injected in this specimen, but the left hepatic arterial bed was partially filled through arterio-arterial anastomoses at the hilum. × 0.44.

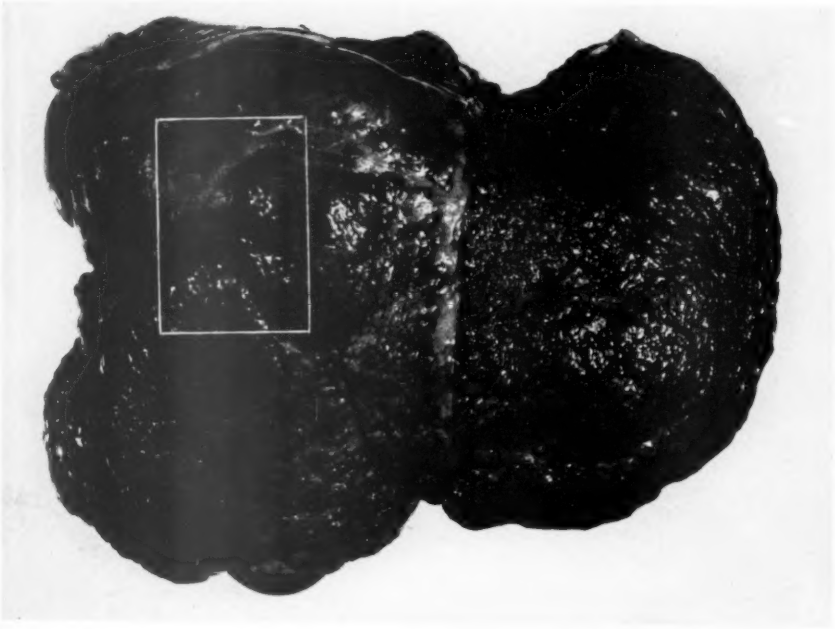


7

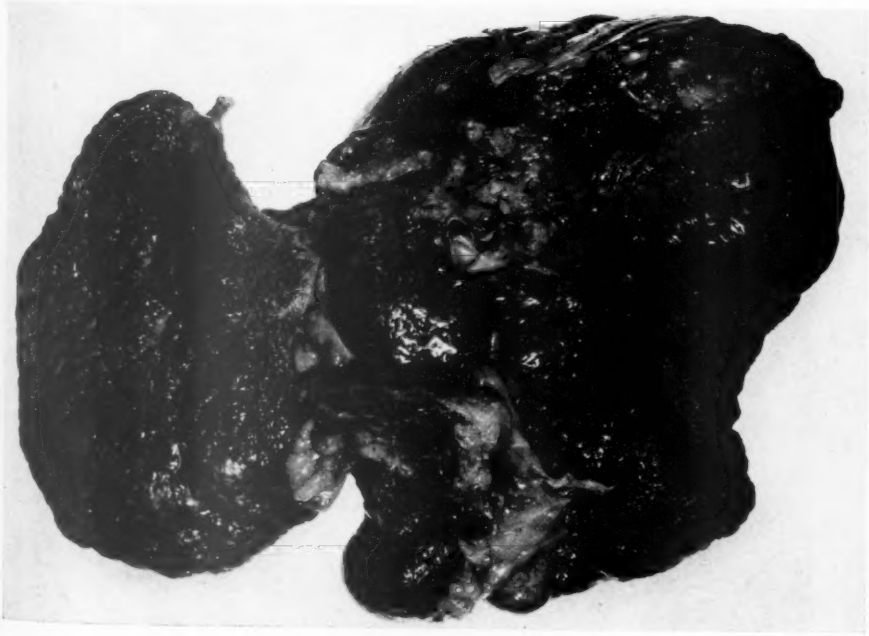
8

FIG. 9. Cirrhotic liver with focal broad scars. The portion outlined is illustrated in Figure 5, after injection and corrosion. Distorted hepatic veins were demonstrated among regenerated pseudolobules, but only portal veins and hepatic arteries could be injected in the broad scars which were almost devoid of parenchyma. × 0.39.

FIG. 10. Posterior surface of a cirrhotic liver with a depressed scar at the right margin. Histologic sections from the scar revealed only a few widely separated, tiny nests of hepatic cells. In the cast from this liver (Fig. 6) hepatic veins were essentially absent from the scar, but portal veins were numerous and compacted there. × 0.4.



9



10

FIG. 11. Anterior inferior surface of the left lobe of a liver with portal cirrhosis. The vessel indicated by the arrow is a large patent umbilical vein. Hepatic arteries (black) are reduplicated, tortuous and enlarged, and many tiny branches have been injected. Portal (gray) and hepatic veins (white) are distorted and reduced. × 1.2.

FIG. 12. Distorted hepatic venous twig from the cast of a cirrhotic liver. Segments of the vessel have been abruptly flattened, narrowed and displaced. The normal regular pattern of arborization has been lost. Only a few small, stunted and distorted tributaries persist. × 4.8.

11

12

FIG. 13. Posterior view of the cast of a cirrhotic liver. A large umbilical vein communicates with the left portal vein. Two smaller para-umbilical veins also enter the falciform ligament from the sharp margin of the right lobe. Portal (gray) and hepatic veins (white) are reduced in number and are greatly distorted. × 0.48.

FIG. 14. Partially trimmed segment from the cast of a liver with portal cirrhosis. A large para-umbilical vein enters the falciform ligament with an S-shaped bend after receiving several tributaries from distal branches of the right portal vein. Such collaterals were surprisingly large and numerous in the cirrhotic livers examined. The tiny, nonbranching vessel to the right is a patent umbilical vein. Note the plexus of enlarged hepatic arteries (black) along each portal vein (gray). × 1.5.

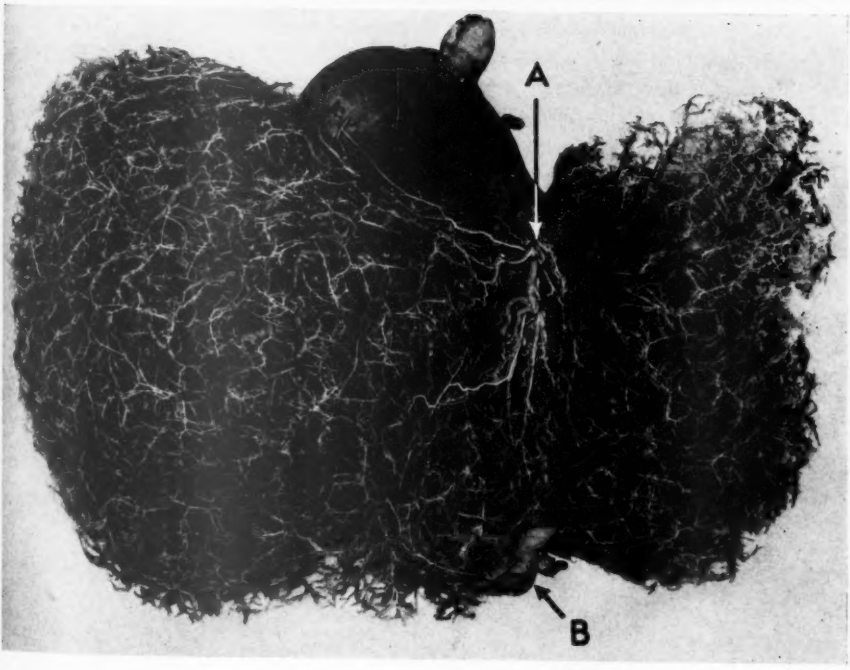
13

14

FIG. 15. Portion of the cast of a liver with "thyrotoxic" cirrhosis. A large looping anastomosis communicates between a portal vein on the left and a hepatic vein at the right center. Large venovenous anastomoses such as this were uncommon in cirrhotic livers. × 2.5.

FIG. 16. Cast of a liver with severe congestive (cardiac) cirrhosis from a patient with congenital tricuspid insufficiency. The inferior vena cava and the hepatic veins are tremendously dilated, and both portal and hepatic veins are distorted. The arteries, appearing almost white in the illustration, are enlarged. At "A" diaphragmatic arteries and veins have been injected in retrograde manner through anastomoses with hepatic arteries and veins. At "B" is seen a large collateral para-umbilical vein arising from distal branches of portal veins. × 0.45.

15



16

NODULAR REGENERATIVE HYPERPLASIA OF THE LIVER*

PAUL E. STEINER, M.D.

From the Institute for Cancer Research and Department of Pathology, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

The purpose of this communication is to emphasize that hepatocytic hyperplasia or regeneration may take place in the absence of fibrous tissue proliferation, that it may be nodular, and that the resulting lesion, here called nodular regenerative hyperplasia, may simulate cirrhosis without, however, meeting the precise criteria and definition of cirrhosis. This condition is not cirrhosis, does not necessarily lead to cirrhosis, has a different significance from that lesion, and ought not be confused with it if statistics are to be meaningful.

For distinction and discussion in this paper, cirrhosis may be defined as a pathologic process in the liver, characterized by nodular hyperplasia or regeneration plus a fibrous tissue increase in the form of scars, bands, membranes or septums, both components together resulting in distortion of the normal lobular architecture. The normal lobular pattern is altered by the combined effects of the two abnormal processes, and both components are essential by definition. This is in agreement with opinions voiced at the Havana[1] and Kampala[2] conferences.

Nodular regenerative hyperplasia, as here described, fails to meet that definition of cirrhosis because it lacks the fibrous bands, scars or septums. On the other hand, the fibrous tissue may be increased in a liver without appreciable parenchymal hyperplasia, and this condition, designated hepatofibrosis, also is not cirrhosis. That is not to deny that these two conditions, nodular regeneration and hepatofibrosis, are important.

The essential features of nodular regenerative hyperplasia are illustrated in a series of cases by Figures 1 to 12. The nodular appearance of such livers is shown by Figures 1 to 7. Higher magnifications of some nodules in Figures 3 to 7 show that this nodularity is due to liver cell proliferation, and this fact is verified also by the higher power views in Figures 8 and 9. The absence of new fibrous tissue and of fibrous scars or septums is shown in Figures 1 to 7 and 10 to 12; only pseudo-septums are present. They are false in the sense that they are composed of structures other than connective tissue.

Stains for connective tissue and for reticulum were made in some of these cases. New collagen was not formed about the nodules. The

* Aided by a research grant from the National Cancer Institute (C-3251).
Received for publication, February 7, 1959.

reticulum fibers around the nodules showed dislocation and a relative increase because of atrophy of the parenchyma and compression. With experience and thin well-stained sections, the condition can be recognized and distinguished from cirrhosis without stains for fibrils.

Regions in which atrophy of liver cells (Figs. 10 to 12) or necrosis of liver cells (Fig. 12) had occurred may display some degree of condensation of the pre-existing stromal elements without this fibrillar substance becoming compact or fused. When such regions are linear, they may falsely simulate the septums or bands of cirrhosis. Such suggestions of cirrhosis are also caused or enhanced by the compressive effects of the expanding regenerated nodules of liver cells distorting or displacing normal anatomic structures such as portal tracts or central veins (Fig. 11). The resulting alterations appear nodular, and may be mistaken for cirrhosis unless a more precise analysis is made.

In nodular regenerative hyperplasia the nodules are small, usually less than an anatomic lobule in size, and one or more to the lobule. Rarely are they larger than a liver lobule. Grossly, a slight degree of irregularity of the lobular architecture is usually visible, as well as an accentuation of the lobules. The condition is present throughout the liver, which is smaller on the average than normal. The appearance is suggested by the original diagnoses which were mild cirrhosis or chronic passive congestion.

The nodules are recognized as being composed of new liver cells by their abnormal relation to the portal tracts and the veins (Figs. 4 to 11), irregularity of the liver cords, enlargement of the liver cells, great frequency of binucleate cells, occasional presence of mitotic figures, and by the evidences at the margins of the compressive effects of expanding nodules (Figs. 1 to 7 and 10 to 12).

The only serious confusion in differential diagnosis is with the appearance in a section marginal to a portal area in a liver which has also a severe grade of passive congestion. There is no problem in differential diagnosis when the plane of the section strikes the portal tract because the typical lesion of central passive congestion is then easily recognized.

In regenerative nodular hyperplasia, band-like zones, mimicking the fibrous bands, septums, or membranes of cirrhosis, may be produced by the compressive effects of expanding nodules without any fibrous increase having occurred. In such regions atrophy or the actual loss of liver cells, whether by a process of atrophy or necrosis, permits the pre-existing fibrillar stroma to come closer together. Partial collapse leads to a relative increase of stroma in the area, but

the fibrils are not fused, and many remain separated by epithelial cells. Viewed uncritically, such a zone may suggest the fibrous band of true cirrhosis and lead to this diagnosis.

It is true that with the complete and final disappearance of parenchymal cells from such an area, the resulting collapse permits juxtaposition of the fibrillar framework and its eventual fusion to form a fibrous band or septum. By accretion and the formation of additional collagen, this band may grow, as it does in cirrhosis. Thus, cirrhosis may develop from nodular hyperplasia if the causative factors persist long enough. On the other hand, the condensed band-like area in nodular hyperplasia may re-expand by regeneration of its hepatocytes just as it does in the regeneration which often follows in the areas of collapse accompanying severe central passive congestion.

Nodular hyperplasia is not in itself a progressive lesion. Only if liver injury, which precedes it, is repeated and severe do the effects produce a fibrous increase and, thus, cirrhosis.

The cause of this lesion is liver damage; it represents a reparative, proliferative phase which is nodular. The livers were slightly below average weight, indicating loss of hepatic substance which had not yet been fully compensated by regeneration.

The injury which induces this nodular type of hyperplasia or regeneration is probably irregular in its distribution within the lobule. The stimulus to regeneration produced by the insufficient liver volume consequent to partial hepatectomy leads to uniformly enlarged anatomic lobules.

Nodular hyperplasia, of the type here described, is not rare. It has been possible without difficulty to accumulate about a dozen specimens in necropsy collections examined in Africa in 1957 and at the University of Chicago. Its frequency has not been systematically determined; it would vary with the frequency of the ailments which it accompanies. It has been found associated with a variety of major diseases, the commonest of which have been the passive congestion of severe heart failure and tuberculosis. In no instance was hepatic abnormality recognized clinically.

There is no reason, statistical or otherwise, to believe from the nature of the major disease with which nodular regenerative hyperplasia was associated that it was the early stage of the common forms of cirrhosis. At the same time it is probable that some of the cases, with repetition of the insults, might have developed the special pattern of central cardiac cirrhosis. From what is known of the etiology and pathogenesis of the two commonest varieties of cirrhosis, namely, postnecrotic and portal cirrhosis, nodular regenerative hyperplasia as here

described is not necessarily a way station in their development. It is, therefore, not called "early" cirrhosis.

By the use of criteria and standards which were less precise than those here defined and advocated, some of these cases had earlier been diagnosed as mild or "early" cirrhosis. To regard them as such is now believed to be undesirable, inasmuch as their significance is different and they would confuse the thinking on the pathogenesis of true cirrhosis. Probably only those cases occurring in cardiac decompensation would ever have progressed to cirrhosis, and that of the ambiguous central lobular type. Until the situation is clarified, it is probably best to retain nodular regenerative hyperplasia without fibrosis as a category separate from the cirrhosis and the hepatofibrosis groups. The statistics on cirrhosis would be more meaningful, precise, and comparable if the cases of nodular hyperplasia and hepatofibrosis were kept in separate categories.

Edmondson has emphasized the condition of "multiple nodular hyperplasia" as it is seen in cirrhotic livers.[3] The nodules are sometimes large. By definition, this condition may be accepted as, in greater or less degree, a regular part of the lesion of cirrhosis.

Summary

Nodular regenerative hyperplasia of the liver is characterized by innumerable small areas of focal regeneration more or less distinctively demarcated by adjacent compressive, atrophic, other degenerative, and circulatory effects. Lacking a true increase in fibrous tissue, the lesion does not conform to the definition of cirrhosis, which it may sometimes resemble. It represents a regenerative phase, after parenchymal damage of local character, within the lobules but generalized throughout the liver.

It was found fairly commonly in damaged livers associated with a wide variety of conditions, the commonest of which was passive hyperemia of cardiac decompensation. It is not in itself a progressive lesion, and is not "early" cirrhosis. If the causative factors for the hepatic damage persist, it is conceivable that cirrhosis of the central or cardiac type may develop, but the lesion is not an early stage of portal or postnecrotic cirrhosis. Statistics on cirrhosis would be more accurate, meaningful, and comparable if cases of nodular regenerative hyperplasia were classified separately.

REFERENCES

1. Fifth Pan-American Congress of Gastroenterology, La Habana, Cuba, January 20-27, 1956. Report of the Board for Classification and Nomenclature of Cirrhosis of the Liver. Sherlock, S. (chairman). *Gastroenterology*, 1956, 31, 213–216.

2. Symposium in Kampala, August 1956. Cancer of the Liver Among African Negroes. *Acta Unio internat. contra Cancrum,* 1957, **13**, 519–522.

3. Edmondson, H. A. Tumor Lesions: Multiple Nodular Hyperplasia. In: Atlas of Tumor Pathology, Section VII, Fascicle 25, Tumors of the Liver and Intrahepatic Bile Ducts, pp. 191–192, Subcommittee on Oncology of the Committee on Pathology of the National Research Council, Armed Forces Institute of Pathology, Washington, D.C., 1958.

[*Illustrations follow*]

LEGENDS FOR FIGURES

All sections were stained with hematoxylin and eosin.

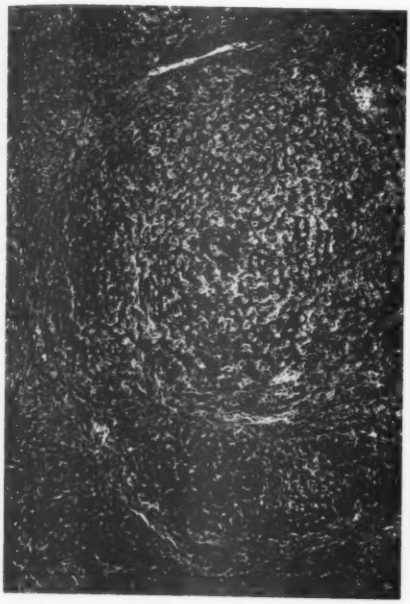
Figures 1 to 3 show the low power appearances of the liver in 3 cases.

FIG. 1. One distinct regenerative nodule and others that are less distinct, separated by pseudo-septums of several types. Case 1. Man, age 87. Liver weight, 1,300 gm.; finely granular. × 42.

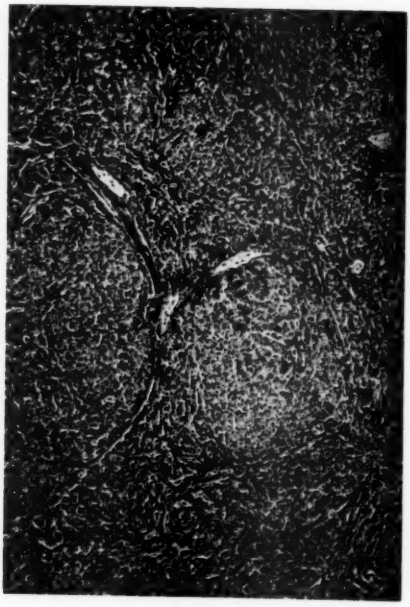
FIG. 2. The regenerative nodules are small but clearly seen because of their central large pale cells. The pseudo-septums are poorly developed. Case 3. Woman, age 71, died of complications after resection of a cecal tumor. Liver weight, 1,550 gm.; had irregular lobules. × 42.

FIG. 3. Portions of several nodules distinctly demarcated by atrophic and congested zones but without increase in fibrous tissue. Case 4. Woman, age 65, died after repeated episodes of cardiac decompensation with coronary occlusion. Liver weight, 1,630 gm.; lobules irregular in size. × 42.

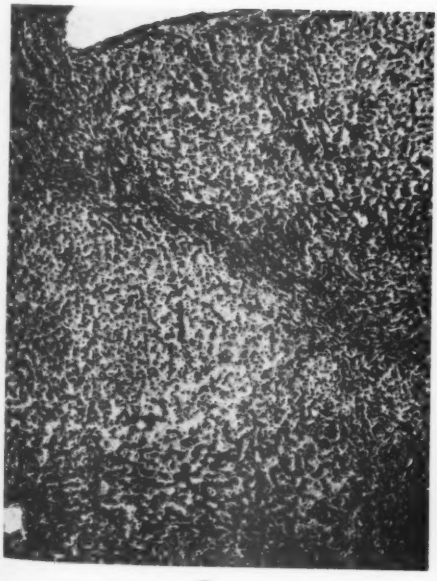
FIG. 4. Distinct regenerative nodule adjacent to a small portal tract, demarcated by a zone of atrophy and hyperemia. Case 1. × 85.



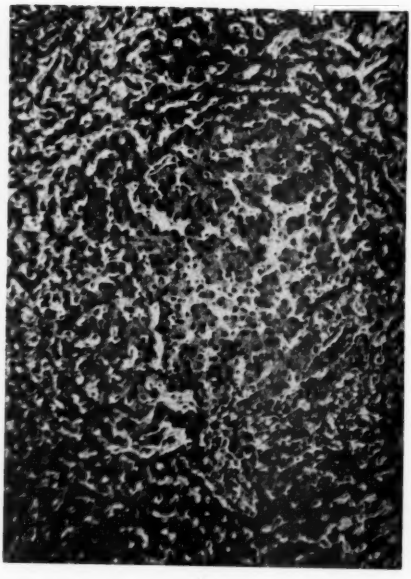
1



2



3



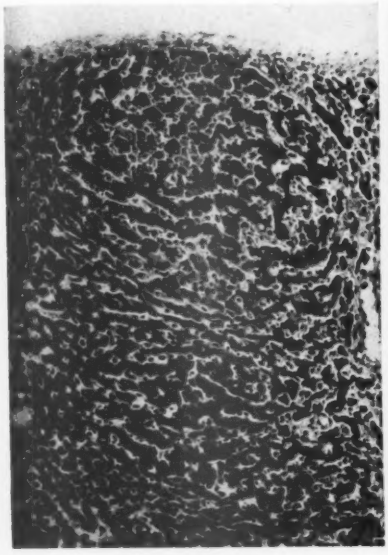
4

FIG. 5. Here the regenerative nodule is subcapsular, although others were present elsewhere. Adjacent compression effects and atrophy are distinct. Case 6. Man, age 56, died of Whipple's intestinal lipodystrophy. Liver weight, 1,720 gm.; anatomic lobules were not grossly visible. × 75.

FIG. 6. The regenerative nodule is adjacent to a small central vein, seen above. Compression effects and atrophy of adjacent liver cords are evident. Case 5. Man, age 20, died after many bouts of decompensation; chronic rheumatic valvular disease. × 75.

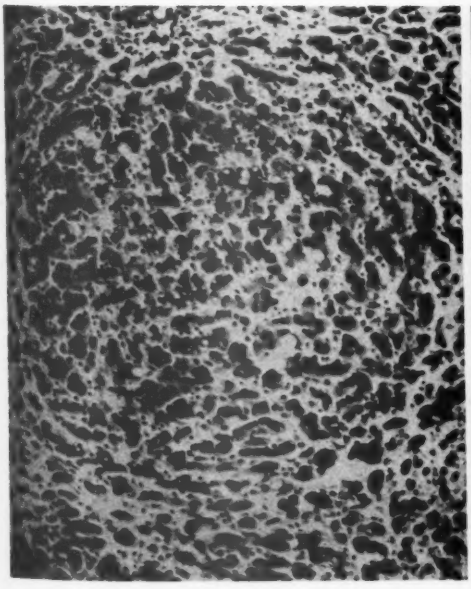
FIG. 7. One distinct nodule and margins of others, set off by compressive and atrophic alterations. Woman, age 62. × 110.

FIG. 8. A high power view within nodule to show atypical plump cords, enlarged liver cells, and frequent binucleate cells found in new hepatic tissue. Hyperchromatism and enlargement of many nuclei may be seen. Case 4. × 365.

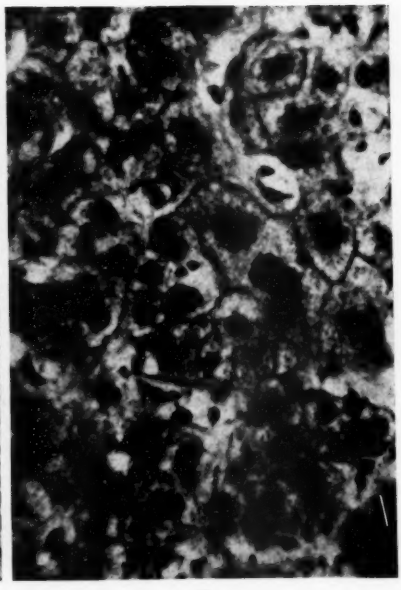


5

6



7



8

FIG. 9. Another high power view within nodule, showing atypical plump cords, enlarged liver cells, and frequent binucleate cells. Case 1. × 365.

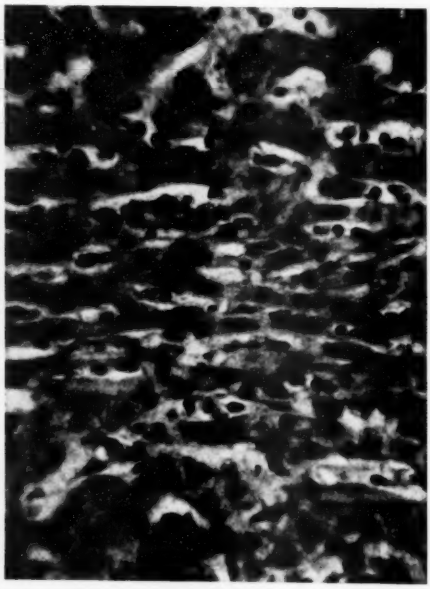
Figures 10 to 12 illustrate in greater detail the zones between the nodules which falsely produce the appearance of septums or bands.

FIG. 10. A distinct band-like zone consists chiefly of greatly compressed liver cords with atrophic cells. Case 2. × 322.

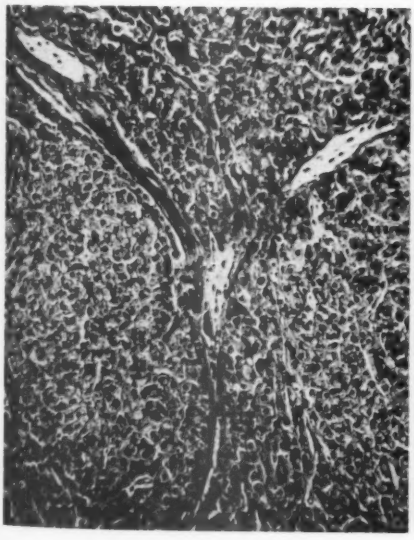
FIG. 11. A branching structure, which separates regenerative nodules, consists of displaced and compressed portal and central structures and their surrounding supporting tissues. There is here no new formation of fibrous tissue. Case 3. × 85.

FIG. 12. The band-like zone at the margins of a distinct nodule is composed of collapsed hepatic stroma and leukocytes; hepatocytes have disappeared. Special stains showed condensation of pre-existing fibrillar structures in such areas, with a relative increase but no recognizable new formation. Case 8. × 322.

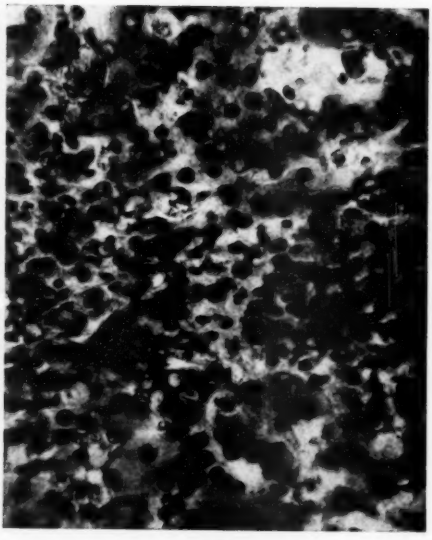
9



10



11



12

SIGNIFICANCE OF HEMATOXYLIN BODIES IN THE NECROPSY DIAGNOSIS OF SYSTEMIC LUPUS ERYTHEMATOSUS *

JOHN W. WORTHINGTON, JR., M.D.†; ARCHIE H. BAGGENSTOSS, M.D., and MALCOLM M. HARGRAVES, M.D.

From the Section of Pathologic Anatomy and the Section of Medicine, Mayo Clinic and Mayo Foundation, Rochester, Minn.

Systemic lupus erythematosus is commonly characterized by diffuse and nonspecific manifestations. Often the symptoms are vague and the physical findings minimal even in patients who are obviously critically ill. The clinical diagnosis without the demonstration of the lupus erythematosus (LE) cell is frequently a difficult one to establish. At necropsy also, the observations may be minimal and nonspecific despite a prolonged illness, and often the diagnosis cannot be established on a morphologic basis alone.[1] The LE cell and the LE cell phenomenon clinically provide what is generally considered a highly specific test for systemic lupus erythematosus. According to published reports, the hematoxylin body possesses the same significance in necropsy material.[2,3]

Hematoxylin bodies are reddish-purple, amorphous, usually ovoid or spindle-shaped; each is approximately the size of a nucleus. They are found in the tissues, where they occur singly (Figs. 1 and 2), in clusters, and, at times, coalesced to form masses (Fig. 3). Histochemical studies have shown that the hematoxylin body is derived from nuclear substance and that it is very similar to the inclusion body of the LE cell.[3,4] In the formation from nuclei, the alterations appear primarily to involve nucleoproteins, rather than nucleic acids as was thought earlier.[5]

The bodies were initially identified and described by Gross,[6,7] who observed them in cardiac lesions and considered them to be characteristic of and probably specific for lupus erythematosus. Klemperer and associates,[3] the first to report an extensive study of hematoxylin bodies, found them in 32 of 35 cases of lupus erythematosus. In a control group they found none except in one case of scleroderma which also showed the lesions of lupus erythematosus. They concluded that free hematoxylin bodies were specific structural features of lupus erythematosus and that they originated in a characteristic alteration of

* Abridgment of a thesis submitted by Dr. Worthington to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

Received for publication, December 15, 1958.

† Fellow in Medicine, Mayo Foundation. The Mayo Foundation is a part of the Graduate School of the University of Minnesota.

nuclei of mesenchymal cells. Gueft and Laufer[2] found hematoxylin bodies in 13 of 14 cases of systemic lupus erythematosus, but did not find them in renal and ovarian tissue from 50 control cases. They concluded that the bodies constituted a reliable criterion for the diagnosis of systemic lupus erythematosus in necropsy tissues.

Differing opinions concerning the significance and incidence of hematoxylin bodies have been expressed. Ross and Wells,[8] in a review of the literature in 1953, stated that their significance had not finally been determined. Teilum and Poulsen[9] were able to find them in only 6 of 15 cases of disseminated lupus erythematosus, and their detection usually required prolonged careful search. Dubois,[10] using the term "hematoxylin bodies" to include both the bodies in the tissues and the lysed nuclear masses found in LE cell preparations, stated that their presence was highly suggestive of the disease, but not diagnostic, as similar alterations might be encountered in scleroderma and hyperglobulinemia. Worken and Pearson[11] reported the only documented case of which we are aware, in which hematoxylin bodies were found in a patient not believed to have lupus erythematosus.

In view of the potential significance of a pathognomonic necropsy lesion in systemic lupus erythematosus, and the lack of full agreement as to the significance of hematoxylin bodies as expressed in current literature, the present study was undertaken to evaluate further the diagnostic significance of these bodies.

Material and Method

Twenty consecutive necropsy cases encountered between 1950 and 1955 which met certain diagnostic requirements for systemic lupus erythematosus were investigated. The minimal diagnostic requirements for inclusion of a case were that it meet at least 2 of the following 3 specifications: (a) a clinical history supportive of the diagnosis; (b) necropsy findings supportive of the diagnosis; and (c) the presence of LE cells in peripheral blood or bone marrow preparations. Material utilized included the clinical history, necropsy protocols, and histologic sections of all the organs except the brain. Sections of tissue fixed in 10 per cent neutral formalin, cut at 7 μ and stained with hematoxylin and eosin were examined. Feulgen, Mallory-Heidenhain, von Kóssa and other special stains were used when indicated. Hematoxylin and eosin and Feulgen stains of consecutive tissue slices were utilized in the investigation of agglomerates and masses. The histologic sections were examined under an oil-immersion-lens system at a magnification

of 970 or under a high quality dry lens system at a magnification of 600. The "high-dry" lens system with a magnification of 430 as found on most medical microscopes was not adequate for consistent differentiation of single hematoxylin bodies from simulative material.

Tissues from 50 selected control cases were examined. The controls included a minimum of 4 cases with each of the following conditions: bacterial endocarditis; acute, subacute and chronic glomerulonephritis; periarteritis nodosa; generalized scleroderma; dermatomyositis; rheumatoid arthritis; and acute rheumatic carditis. Control sections were of the heart and kidney, which were selected because they manifest a high incidence of hematoxylin bodies in lupus erythematosus,[2,3] are often extensively affected in diseases that may resemble systemic lupus erythematosus, and frequently contain degenerative and necrotic material resembling hematoxylin bodies. A section cut to include a cross section of the posterior mitral valve leaflet, valve ring, atrial wall and ventricular wall was examined at a magnification of 970 or 600. Perivascular areas and lesions in the myocardium were investigated in the same manner. Tissue from the kidney was examined under similar magnification until 60 glomeruli had been encountered.

Additional control material consisted of sections from necrotic lymph nodes associated with various diseases, of tissue selected to show varying types of necrosis, and of 20 routine necropsy cases taken at random.

In a preliminary investigation based on all available descriptions[2,3,6,12-14] much difficulty was encountered at times in distinguishing single hematoxylin bodies from simulative material. The greatest difficulty encountered was in distinguishing them from fragments resulting from other types of nuclear breakdown, particularly karyolysis. Several instances of homogeneous nuclear-sized masses indistinguishable from single hematoxylin bodies were found in areas of gross necrosis in other conditions (Fig. 4). Certain other material, also primarily in areas of necrosis, could be distinguished only with considerable difficulty. Although results of histochemical studies are known, hematoxylin bodies are still recognized primarily by their morphologic characteristics and their staining reaction with hematoxylin and eosin. On the basis of the difficulties encountered in the preliminary investigation, precise criteria for the identification and differentiation of hematoxylin bodies were established. Emphasis was placed on exclusion of all material that might simulate hematoxylin bodies and produce false positive results when used as a diagnostic feature.

Criteria for Hematoxylin Bodies Occurring Singly and in Small Groups

The size of the single hematoxylin body is approximately that of a cell or a nucleus. It is usually round or oval, but not necessarily so. Its color when stained with hematoxylin and eosin is variable and may range from purplish red to pinkish blue. It must be amorphous and free from chromatin dots or particles. Some mottling is permissible, the chromatin at times having an "ironed-out" appearance. It must be free of cytoplasm. It is translucent and generally less dense than the normal nuclei in the field. It must have a reasonably defined nonrefractile border. To exclude similarly staining cytoplasm, it must be distinct from the cytoplasm of adjacent cells. The single bodies and small groups of up to 6 or 8 component single bodies must not be in an immediate area of necrosis. Nuclear debris must be absent from the immediate area, and nuclei with well defined, distinct chromatin patterns must be present in the area. (This requirement was designed to eliminate much of the problem of distinguishing the single hematoxylin body from a nucleus undergoing karyolysis.)

Criteria for Hematoxylin Bodies Occurring as Clusters, Agglomerates, or Masses

Large hematoxylin bodies occurring as clusters, agglomerates, or fused masses of single bodies may vary in size from small clumps to masses several hundred microns in diameter. They may and often do occur in areas of necrosis. A lower limit in size for acceptable aggregates of hematoxylin bodies occurring in areas of necrosis is set at the equivalent of 6 to 8 nuclei. The masses must have no structural pattern but need not be homogeneous. They are relatively dense, and are purple to purplish red when stained with hematoxylin and eosin. Adjacent and identifiable component single bodies must meet all the requirements for single bodies other than that relating to presence in an area of necrosis. If transitions from nuclei or single hematoxylin bodies to clumps and masses cannot be traced in the given organ, the masses must either take the Feulgen stain or must not take stains used to demonstrate calcium.

Results

Hematoxylin bodies were found in 2 or more organs in 17 of the 20 cases of systemic lupus erythematosus. They were present in widely scattered locations (Table I). In general, they were seen most frequently at sites where fibrinoid alteration of the collagen was associated with a mild inflammatory reaction.

TABLE I

Frequency and Distribution of Hematoxylin Bodies in 20 Consecutive Necropsy Cases of Systemic Lupus Erythematosus

	Case																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Age, years:	16	47	21	30	25	23	16	14	31	20	27	21	35	32	18	47	4	25	14	20
Mitral, post.*	+	+	+	+		+	+		+	+	+				+	+				+
Tricuspid, post.*	+			+		+	+		+		+	+		+	+	+			+	
Endocardium (other)				+		+	+		+											
Myocardium		+	+			+						+				+				
Epicardium						+				+		+								
Kidney		+	+			+	+	+			+			+	+	+	+		+	+
Ovary	+	+	+	+		+		+						+	+					+
Lymph nodes				+		+			+	+					+					+
Lung	+	+		+		+														
Synovial membrane																	+		+	+
Spleen	+			+					+							+				
Adrenal							+					+		+						
Pleura				+					+			+								
Peritoneum	+					+											+			
Skin						+			+											
Liver				+																+
Uterus		+																		
Urinary bladder		+																		
Aorta												+								
Thyroid		+																		
Esophagus														+						
Breast																				+
Pancreas								+												

* Includes posterior leaflet of valve, valve ring and adjacent endocardium.

The greatest incidence of hematoxylin bodies was in the heart, where they were present in 15 cases. Here they were found primarily in the endocardium and were encountered most frequently at the valve ring and in adjacent portions of valvular and mural endocardium (Fig. 1). They occurred singly, in clusters, in wavelike strata, and in agglomerates. Most often seen were scattered single hematoxylin bodies, the large agglomerates being found in only 3 cases, in which they occurred in portions of valves showing valvulitis. Six of the cases were characterized by nonbacterial verrucal endocarditis of the Libman-Sacks type; hematoxylin bodies were found in all these cases.

The bodies were found in the kidney in 12 of the 20 cases. They occurred most frequently as single bodies in the lumens of the glomerular capillaries. Other sites of involvement included the walls of vessels and the glomerular tuft near the hilus; here they occurred singly or as small agglomerates. They were rarely encountered in the interstitial tissues. While occasionally hematoxylin bodies could be readily found in many glomeruli, they were generally found only after careful, prolonged search of many glomeruli. "Wire-loop" thickening of the glomerular basement membrane was observed in 13 cases. Hyaline thrombi were occasionally seen in the glomerular capillary lumens, and at times they showed a slight basophilic staining. The resultant appearance was suggestive of a pale-staining hematoxylin body. Focal loop necrosis of the glomeruli was present in 5 cases. Destruction of nuclei in the involved loop was usually present, as evidenced by nuclear debris. Hematoxylin bodies were occasionally found as small clusters in the areas of focal necrosis. Single hematoxylin bodies also undoubtedly were present, but because of the uncertainty in distinguishing them from other types of lysed nuclei, they were excluded by the criteria used in the study. The bodies, while present in kidneys showing minimal or no evidence of other involvement, were more readily found in kidneys showing extensive anatomic change.

Hematoxylin bodies were found in the ovary in 8 cases. They occurred almost exclusively as scattered single bodies in the walls of arteries and arterioles (Fig. 2). The lymph nodes contained them in 6 instances. In 3 of these, they were present as large purplish masses filling dilated sinuses and occurring in zones of extensive necrobiosis (Fig. 3A). The large masses were surrounded in each instance by smaller agglomerates and by single hematoxylin bodies (Fig. 3B). Although the large masses were striking in appearance when seen, more frequent was a single hematoxylin body occurring usually in the capsular tissue in lymph nodes that showed little evidence of other change. The bodies were seen occasionally in the walls of pulmonary

vessels and in the pleural and subpleural tissue. They were infrequently observed in the spleen. The laminated periarterial "onion-skin" fibrosis often present in the spleen in lupus was observed in 15 cases. In the sections of bone marrow, hematoxylin bodies were conspicuous by their complete absence; in each case, multiple sections of both vertebral and sternal marrow were examined.

Where hematoxylin bodies were numerous, cells showing similar nuclear changes, namely, homogenization and altered staining, were often encountered. Histiocytes, lymphocytes, neutrophils, and fibrocytes could be identified as showing such changes. As had been observed previously,[3] only mesenchymal cells were so affected.

In general, the greatest number and widest distribution of hematoxylin bodies encountered occurred in the cases which were the most typical of lupus erythematosus both clinically and pathologically, whereas a smaller number, or absence, of the bodies was observed in the less typical and the atypical cases. There was no apparent correlation between the presence of the bodies and the age of the patient or duration of illness. The ages of the patients ranged from 4 to 47 years, the majority being in the second and third decades of life. All were female. All but one had been treated with cortisone or related drugs. LE cells had been demonstrated in 18 cases and had been searched for in all 20. Multiple hematoxylin bodies were found in one case in which the search for LE cells had been unsuccessful on 3 occasions, but no hematoxylin bodies were found in the second case in which search for LE cells had been unsuccessful. LE cells had been demonstrated during life in 2 of the 3 cases in which hematoxylin bodies were not found.

In 3 of the 70 control cases, material was present that could not be distinguished from a single hematoxylin body. In the first of these cases, there was generalized scleroderma with typical skin changes and extensive renal involvement, including thickening of the glomerular basement membrane, "wire loops," and areas of infarction. A single nuclear-sized, purplish-red mass, indistinguishable from a hematoxylin body, was found in a glomerulus. Several cells seen in sections of the kidney from this case had reddish, somewhat homogeneous nuclei, but only the one mass meeting all the requirements for a hematoxylin body was found in a search of all organs. The heart and spleen contained no lesions suggestive of systemic lupus erythematosus. In the second control case, one of portal cirrhosis, one mass indistinguishable from a single hematoxylin body was found in the endocardium near the base of the mitral valve. No other lesions were present in the area, and no nuclei were seen showing the changes characteristic

in the development of hematoxylin bodies. In the third control case, one of subacute glomerulonephritis and multiple myeloma, a similar single mass without accompanying alterations was found in the same location as in the second case. Further study of the last two cases revealed no additional lesions of this type, and nothing indicative of systemic lupus erythematosus was found in the clinical record or among the other pathologic features. Material closely resembling and at times indistinguishable from single hematoxylin bodies was found on several occasions in areas of necrosis, especially in cases of endocarditis and periarteritis. These bodies were ruled out on the basis of their occurrence in areas of necrosis. In the control cases nothing was encountered except calcium deposits which resembled clusters or agglomerates of hematoxylin bodies, regardless of the degree of necrosis or of other changes in the tissue.

Comment

The basic morphologic characteristics of the single hematoxylin body are that it is amorphous, purplish red, and approximately the size of a nucleus. Since these are nonspecific, the chance occurrence of similar-appearing material might well be expected. In distinguishing single hematoxylin bodies from simulative material, the greatest difficulty encountered was in regard to other types of nuclear breakdown. Homogenization and lysis of the nuclear chromatin,[3,15] forms of nuclear alteration or breakdown, are believed to be an initial step in the formation of the hematoxylin body and the inclusion body of the LE cell. The conventional types of nuclear breakdown include pyknosis, karyorrhexis and karyolysis. Nuclei undergoing pyknosis and karyorrhexis can be readily differentiated from hematoxylin bodies by their dissimilar appearance. Karyolysis presents a greater problem. It, like formation of the hematoxylin body, is also a process of nuclear lysis. In karyolysis as defined in standard textbooks of pathology, the nucleus stains progressively less deeply with basic dyes, though its shape may be retained.[16-18] The cytoplasm may be dissolved by ferments.[16,18] At certain stages in the process of karyolysis, a cell may be present as an altered, essentially homogeneous, bluish-pink staining, nuclear-sized mass that is free from cytoplasm (Figs. 4 and 5). As such, it may be indistinguishable from the free hematoxylin body of lupus erythematosus.

Polychromatophilic erythrocytes may simulate single hematoxylin bodies when they are heavily stained with hematoxylin and lack the usual biconcave shape. The cytoplasm of histiocytes when cut so as not to include the nucleus, precipitated protein such as occurs at sites

of pulmonary edema and in renal tubules, and nuclei in slides with imperfect staining or fixing of the tissue may also be misinterpreted. The large agglomerates and large clusters of single bodies appear to be relatively distinctive in appearance.

The observations that certain material in zones of necrosis may be indistinguishable from hematoxylin bodies and that material indistinguishable from single hematoxylin bodies was encountered in non-necrotic areas in 3 cases without lupus erythematosus cast doubt on the specificity of these elements. It should be emphasized that when viewed from the point of total incidence, frequency, and distribution, the pattern of the bodies as seen in systemic lupus erythematosus is distinctly different from that of similar-appearing material seen in control cases. In the present investigation when hematoxylin bodies were found in an organ in lupus erythematosus, with few exceptions further search revealed additional bodies elsewhere in the same organ and also in other organs. By contrast, the substance found in non-necrotic regions in the 3 control cases was limited to a single cell-sized body in each instance. That encountered in areas of necrosis among the controls was always limited to those regions. The question of the specificity of the hematoxylin body must thus be considered in terms of these observations. If identification of a number of the bodies meeting the criteria used in this study were required, then none of the control cases would be regarded as manifesting hematoxylin bodies.

The morphologic, tinctorial, and histochemical similarity of the hematoxylin body to the lysed nuclear inclusion mass of the LE cell has been pointed out repeatedly. The hypothesis that the LE cell factor is the agent responsible for both the LE cell phenomenon and the formation of the hematoxylin body probably represents the true situation. However, there are certain differences in the features of the two processes. The LE cell phenomenon is an *in vitro* process[19-21] except in certain special circumstances. The hematoxylin body is an *in vivo* process as indicated by its presence in promptly fixed needle-puncture biopsy specimens from the kidney.[22] Godman and associates[5] observed certain differences between histochemical reactions of the lysed nuclear masses of LE cell preparations and those of hematoxylin bodies in the tissues. They indicated that both were of similar origin but that there was secondary alteration of the hematoxylin bodies during their sojourn in the tissues. The lysed nuclear mass in the LE cell preparation has a strong chemotactic attraction for phagocytic cells in supravital preparations.[15] Although often found at sites of inflammatory reaction, the hematoxylin body shows little if any evidence of such attraction for phagocytic cells. The bodies observed in this investigation were

rarely engulfed by cells and seldom were found in close contact with phagocytes. Because of these differences, it seems advisable to reserve the term "hematoxylin body" for the lysed nuclear masses found in the tissues and the term "lupus bodies" as a generic one to cover both the hematoxylin body and the lysed nuclear mass in LE cell preparations.

Probably the major factors pertaining to the incidence of hematoxylin bodies in a given series of cases of systemic lupus erythematosus are the diligence and length of time spent in searching for them. Often they are found only after prolonged, careful search, using high magnification. Other factors undoubtedly play a role. In this series the bodies were more numerous in classic cases although they were also present in less typical cases. Since cortisone and related drugs decrease the number of LE cells, these drugs might also be expected to decrease the number of hematoxylin bodies as well. All but one of the patients in the present group had received cortisone or related drugs, and the incidence of the bodies was only slightly less than in previous groups in which most of the patients had not received these drugs.[2,3] This suggests that the influence of the steroid drugs on the incidence of hematoxylin bodies is small or nonexistent.

The occurrence of hematoxylin bodies in other conditions is reported infrequently. Worken and Pearson[11] found multiple hematoxylin-staining bodies in a 23-year-old man with allergic angiitis. Klemperer and associates[8] observed them in one case of scleroderma which also showed anatomic lesions of systemic lupus erythematosus. Dubois,[10] using the term "hematoxylin body" to include hematoxylin bodies in the tissues and the lysed nuclear masses found in LE cell preparations, stated that hematoxylin bodies could be found in scleroderma and hyperglobulinemia. He did not specify whether the material found in these diseases was that found in the LE cell preparation or in the tissues. For reasons previously given, it seems desirable to reserve the term "hematoxylin body" for the lysed nuclear material found in the tissues.

In the use of the hematoxylin body as a diagnostic feature, the same hazards are encountered as in other methods of cytologic diagnosis. The morphologic characteristics of the single hematoxylin body are not distinctive enough to permit a "one-cell diagnosis." Their appearance when collected in clusters, agglomerates, or masses, however, is distinctive. If the identification of a number of bodies is based upon the criteria recommended, their presence appears to be a highly specific feature in the necropsy diagnosis of systemic lupus erythematosus. Their specificity, when so identified, appears to be greater than any of

the other anatomic features of the disease. This specificity, coupled with its occurrence in at least 85 per cent of cases of lupus erythematosus, makes the hematoxylin body a highly significant and useful diagnostic feature.

Summary

Hematoxylin bodies were found in 17 of 20 consecutive necropsy cases of systemic lupus erythematosus. They were seen most frequently in the presence of classical clinical and pathologic features, but were also encountered in multiple organs even in less typical cases. The single hematoxylin body could not always be distinguished from nuclei undergoing karyolysis or from other infrequently encountered substances with similar appearance. These substances, when observed in other conditions, were located in areas of necrosis or appeared as isolated findings. Criteria for the identification of hematoxylin bodies were designed to distinguish them from such simulative material and were used in this investigation. When a number of bodies meeting these criteria were identified in a given case, their specificity appeared to be of high degree and to be greater than that of any other anatomic feature of the disease. This specificity, coupled with their occurrence in the majority of cases of disseminated lupus erythematosus, indicates that hematoxylin bodies constitute highly significant and useful diagnostic features.

REFERENCES

1. Baggenstoss, A. H. Visceral lesions in disseminated lupus erythematosus. *Proc. Staff Meet. Mayo Clin.*, 1952, **27**, 412–419.
2. Gueft, B., and Laufer, A. Further cytochemical studies in systemic lupus erythematosus. *A. M. A. Arch. Path.*, 1954, **57**, 201–226.
3. Klemperer, P.; Gueft, B.; Lee, S. L.; Leuchtenberger, C., and Pollister, A. W. Cytochemical changes of acute lupus erythematosus. *Arch. Path.*, 1950, **49**, 503–516.
4. Lee, S. L.; Michael, S. R., and Vural, I. L. The L.E. (lupus erythematosus) cell. *Am. J. Med.*, 1951, **10**, 446–451.
5. Godman, G. C.; Deitch, A. D., and Klemperer, P. The composition of the LE and hematoxylin bodies of systemic lupus erythematosus. *Am. J. Path.*, 1958, **34**, 1–23.
6. Gross, L. The Heart in Atypical Verrucous Endocarditis (Libman-Sacks). In: Contributions to the Medical Sciences in Honor of Dr. Emanuel Libman by His Pupils, Friends, and Colleagues. The International Press, New York, 1932, Vol. 2, pp. 527–550.
7. Gross, L. The cardiac lesions in Libman-Sacks disease; with a consideration of its relationship to acute diffuse lupus erythematosus. *Am. J. Path.*, 1940, **16**, 375–408.
8. Ross, S. W., and Wells, B. B. Systemic lupus erythematosus. A review of the literature. *Am. J. Clin. Path.*, 1953, **23**, 139–160.
9. Teilum, G., and Poulsen, H. E. Disseminated lupus erythematosus: Histopathology, morphogenesis, and relation to allergy. *A. M. A. Arch. Path.*, 1957, **64**, 414–425.

10. Dubois, E. L. The effect of the L.E. cell test on the clinical picture of systemic lupus erythematosus. *Ann. Int. Med.*, 1953, **38**, 1265–1294.

11. Worken, B., and Pearson, R. D. Hematoxylin bodies associated with allergic angiitis in absence of lupus erythematosus. *A. M. A. Arch. Path.*, 1953, **56**, 293–300.

12. Ginzler, A. M., and Fox, T. T. Disseminated lupus erythematosus: A cutaneous manifestation of a systemic disease (Libman-Sacks): Report of a case. *Arch. Int. Med.*, 1940, **65**, 26–50.

13. Gueft, B. Depolymerization of nucleic acid in acute disseminated lupus erythematosus. *Arch. Dermat. & Syph.*, 1950, **61**, 892–897.

14. Klemperer, P.; Gueft, B., and Lee, S. Nucleic acid depolymerization in systemic lupus erythematosus. *J. Mt. Sinai Hosp.*, 1949–1950, **16**, 61–62.

15. Hargraves, M. M.; Richmond, H., and Morton, R. Presentation of two bone marrow elements: The "tart" cell and the "L.E." cell. *Proc. Staff Meet. Mayo Clin.*, 1948, **23**, 25–28.

16. Bell, E. T. A Text-book of Pathology. Lea & Febiger, Philadelphia, 1947, ed. 6, p. 134.

17. Karsner, H. T. Human Pathology. J. B. Lippincott Co., Philadelphia, 1955, ed. 8, p. 72.

18. Mallory, F. B. The Principles of Pathologic Histology. W. B. Saunders Co., Philadelphia, 1914, p. 96.

19. Hargraves, M. M. The L.E. cell phenomenon. In: Advances in Internal Medicine. Dock, W., and Snapper, I. (eds.) The Year Book Publishers, Inc., Chicago, 1954, Vol. 6, pp. 133–160.

20. Lee, S. L. Laboratory studies in systemic lupus erythematosus. *A. M. A. Arch. Dermat.*, 1956, **73**, 313–317.

21. Sickley, J. F.; Friedman, I. A.; Feldhake, C., and Schwartz, S. O. *In vivo* demonstration of the lupus erythematosus phenomenon. *J. Lab. & Clin. Med.*, 1955, **46**, 624–627.

22. Muehrcke, R. C.; Kark, R. M.; Pirani, C. L., and Pollak, V. E. Lupus nephritis: A clinical and pathologic study based on renal biopsies. *Medicine*, 1957, **36**, 1–145.

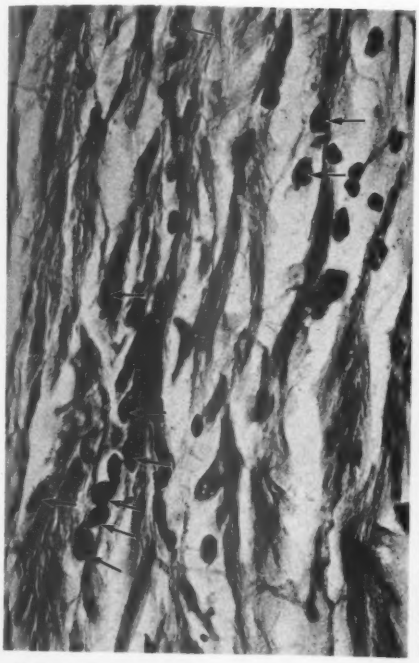
LEGENDS FOR FIGURES

Hematoxylin and eosin stain was used in each of the sections from which the illustrations were prepared.

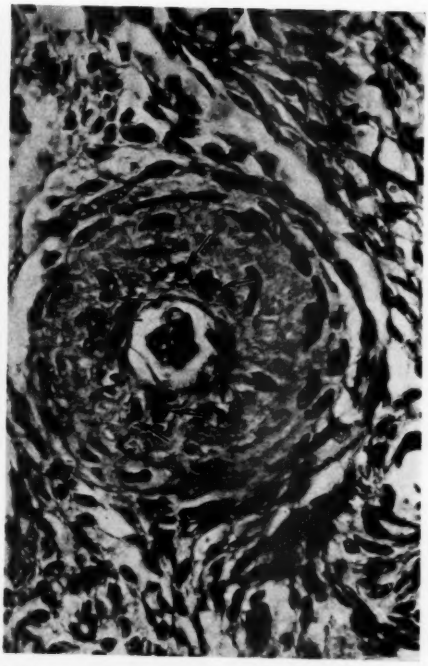
FIG. 1. Multiple single hematoxylin bodies (arrows) in mitral-valve leaflet in a case of systemic lupus erythematosus. Normal fibrocytes, fibrocytes with smudgy, partially "ironed-out" nuclei, and hematoxylin bodies having the shape of fibrocytic nuclei are present. × 600.

FIG. 2. Hematoxylin bodies (arrows) in an ovarian arteriole in a case of systemic lupus erythematosus. × 500.

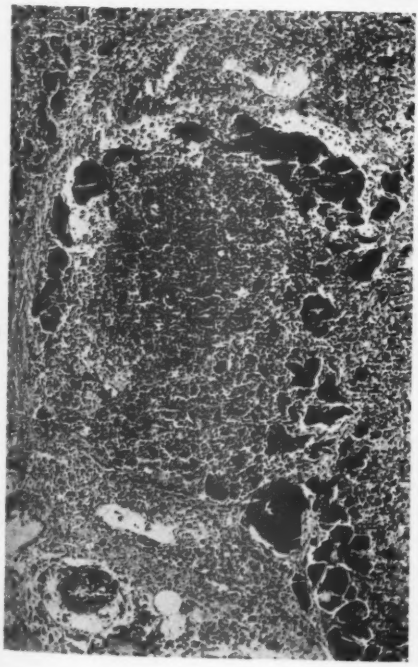
FIG. 3. Lymph node in a case of systemic lupus erythematosus. (A) Hematoxylin bodies occurring as large masses that fill dilated sinuses. × 90. (B) Hematoxylin bodies occurring singly and as smaller masses. Dense pyknotic nuclei and karyorrhectic particles contrast with the smudgy, amorphous, less dense hematoxylin bodies. × 500.



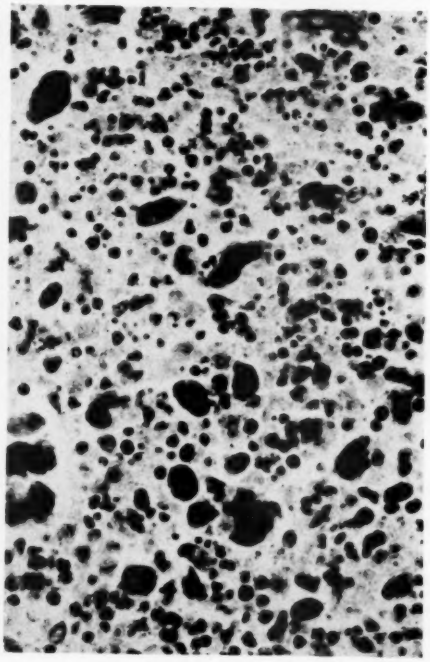
1



2



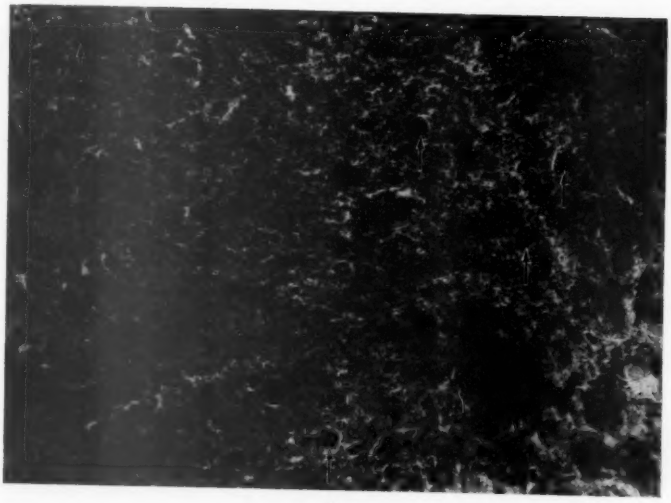
3A



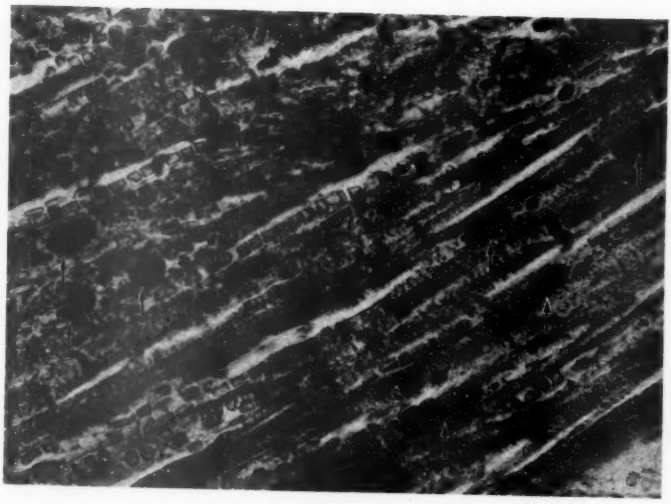
3B

FIG. 4. Valvular vegetation with area of necrosis, showing amorphous, reddish-purple nuclear material (arrows) which resembles hematoxylin bodies in a case of bacterial endocarditis, with no evidence of systemic lupus erythematosus. × 600.

FIG. 5. Myocardium, showing an area of infarction with lysed, swollen, amorphous nuclei (arrows), some of which are free of cytoplasm. These bear marked resemblance to hematoxylin bodies. There was no evidence of systemic lupus erythematosus. × 600.



4



5

STUDIES ON EXPERIMENTAL AMYLOIDOSIS

I. Analysis of Histology and Staining Reactions of Casein-Induced Amyloidosis in the Rabbit*

Alan S. Cohen, M.D.; Evan Calkins, M.D., and Charles I. Levene, M.D.
From the Medical Services, Massachusetts General Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

In the past 35 years amyloidosis has been produced in a number of experimental animals by a variety of techniques. Cattle,[1] horses,[2] rabbits,[3] hamsters,[4] guinea pigs,[5] and mice[6] all have been reported as capable of developing the disease, given the proper stimulus. The latter has consisted of either injection, usually serially, of a variety of substances, or dietary variations, such as cheese supplements or vitamin C deficiency.[5,7] Amyloidosis has also been reported as occurring spontaneously in dogs[1] and in otherwise normal but aged mice of certain strains.[8]

In our laboratory, the induction of amyloidosis in rabbits by means of serial injections of sodium caseinate has provided a reliable and reproducible form of the disorder for study. To date, amyloidosis has been successfully induced in 120 rabbits. Serial bleedings can be carried out with ease and splenic biopsy specimens procured readily. The disease develops eventually to some degree in nearly 100 per cent of injected animals and is histologically similar to the human disorder. The chemical and serologic alterations in the blood of these animals are the subjects of other reports.[9,10]

The present investigation is an analysis of the histologic alterations that occur in different parenchymal organs with the evolution of the disorder. Since a variety of staining techniques have been used in different laboratories in the study of human and experimental amyloidosis, the usefulness of several of these has been compared at the onset and in the final stages of amyloidosis. In addition, fluorescence microscopy and polarization studies have been performed in an effort to characterize amyloid more definitively.

Methods

Thirty-four New Zealand white female rabbits were given subcutaneous injections of 5 ml. of a 10 per cent casein suspension twice

* This is publication No. 253 of the Robert W. Lovett Memorial for the Study of Crippling Diseases. Grants in support of these investigations have been received from the National Institutes of Health, A-1064 (C-2), and the Eli Lilly Company.
Received for publication, February 13, 1959.

weekly, utilizing sterile precautions.* The suspensions were freshly prepared each week from commercially available sodium caseinate (obtained from the Matheson Company, Inc.) suspended in distilled water. No antibiotic agents or other preservatives were added. Periodic cultures revealed that while most of the suspensions were sterile, one occasionally exhibited bacterial growth, especially *Bacillus subtilis* and alpha hemolytic streptococcus. The rabbits were maintained on a diet of standard Purina Rabbit Chow (15 per cent protein) and water was administered *ad libitum*. Eight additional rabbits served as controls.

Individual rabbits were sacrificed at intervals of approximately one month. Tissues were fixed in neutral formalin and embedded in paraffin after alcohol dehydration. Serial sections, 5 μ in thickness, were stained with hematoxylin and eosin, Congo red, van Gieson, methyl violet, and crystal violet stains, and by the periodic acid-Schiff reaction. The tissues of 12 rabbits sacrificed after varying intervals were stained with pyronin methyl green. Unstained sections were examined for autofluorescence, using a Reichert ultraviolet microscope at 365 mμ, and for birefringence, using a Spencer polarizing microscope. Congo red stained sections were also examined for fluorescence and birefringence.

The estimation of the amount of amyloid present in a given case was based on the independent appraisals of two observers, using the hematoxylin and eosin, Congo red, crystal violet and van Gieson stained preparations.

Results

General Incidence

Six rabbits were sacrificed after receiving casein injections twice weekly for one month; none showed any signs of amyloidosis (Table I). Eight rabbits were sacrificed at the end of 2 months; 3 of these had amyloid disease of the spleen. Four of the 6 rabbits sacrificed at the end of 3 months of injections also had amyloidosis, as did all rabbits examined after more prolonged periods of casein injections. In 8 control rabbits, sacrificed at bimonthly intervals, there was no evidence of amyloid.

Gross Appearance and Distribution in Various Organs

As seen in Table I and Text-figure 1, the earliest site of deposition of amyloid was in the spleen. Indeed, all spleen sections from rabbits receiving casein for 4 months or more demonstrated amyloidosis. The

* Many of these rabbits served as controls for various other investigations as yet unpublished. This group of animals includes only 7 rabbits mentioned in our previous report concerning serum changes in amyloidosis.[9]

TABLE I

*Incidence and Extent of Casein-Induced Amyloidosis in 42 Rabbits Examined at Monthly Intervals**

Rabbit no.	Months of casein†	Amount of amyloid‡		
		Spleen	Kidney	Liver
164	0	0	0	0
165	0	0	0	0
182	0	0	0	0
184	0	0	0	0
186	0	0	0	0
188	0	0	0	0
191	0	0	0	0
196	0	0	0	0
120	1	0	0	0
127	1	0	0	0
156	1	0	0	0
171	1	0	0	0
175	1	0	0	0
178	1	0	0	0
98	2	2+	0	0
100	2	2+	0	0
111	2	0	0	0
121	2	0	0	0
122	2	0	0	0
136	2	0	0	0
148	2	1+	0	0
150	2	0	0	0
112	3	4+	4+	2+
116	3	3+	1+	0
132	3	3+	0	0
170	3	2+	0	0
172	3	0	0	0
174	3	0	0	0
67	4	4+	0	1+
115	4	4+	2+	2+
97	5	4+	1+	0
99	5	3+	2+	1+
47	6	2+	2+	0
56	6	4+	2+	0
58	6	3+	4+	0
87	6	4+	4+	0
89	6	3+	3+	0
94	6	4+	2+	1+
92	7	4+	2+	1+
59	9	3+	3+	0
14	10	2+	3+	0
8	12	4+	1+	0

* The first 8 rabbits are controls.

† All time intervals adjusted to the nearest month.

‡ Graded as follows:

0 = no amyloid
1+ = 1 to 25 per cent replacement of the organ
2+ = 26 to 50 per cent replacement
3+ = 51 to 75 per cent replacement
4+ = 76 to 100 per cent replacement

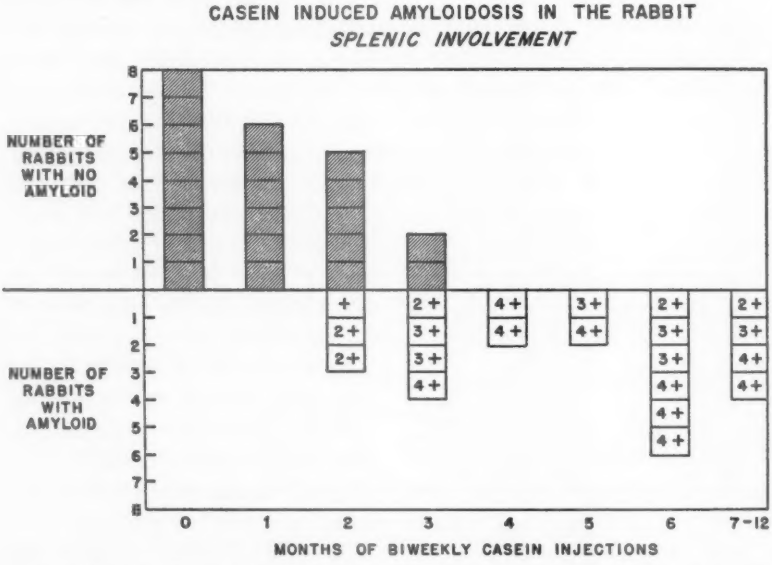
extent of involvement was variable. In several animals receiving the injections for only 3 or 4 months, the spleens showed well over 50 per cent replacement with amyloid. Considerably less was seen in the spleen of one animal which received injections for 10 months.

The normal rabbit spleen was dark red, the weight ranging from 0.5 to 1.5 gm. After one month of injections the spleens were no different in consistency but were slightly larger. The spleens of animals which received casein for longer periods of time and which did develop amyloidosis, were not only larger but firmer and more rubbery. The weight usually was greater with larger amounts of amyloid deposit. For example, one spleen (rabbit No. 100) with 2+ involvement had a weight of 4.2 gm., while that of rabbit No. 99 with 3+ involvement weighed 5.2 gm., and the spleen of rabbit No. 97, with almost total replacement (4+), weighed 6.5 gm.

Renal amyloidosis, primarily glomerular in location, was not found in animals receiving casein for less than 3 months (Text-fig. 2). After 5 months of casein injections, however, renal involvement was invariable. Again, the degree of amyloidosis varied from rabbit to rabbit. One animal, which received casein injections for a full year, exhibited a rather minor degree of renal amyloidosis. Kidneys were weighed in about

one fourth of the cases. No changes in weight were demonstrable with increasing deposition of amyloid.

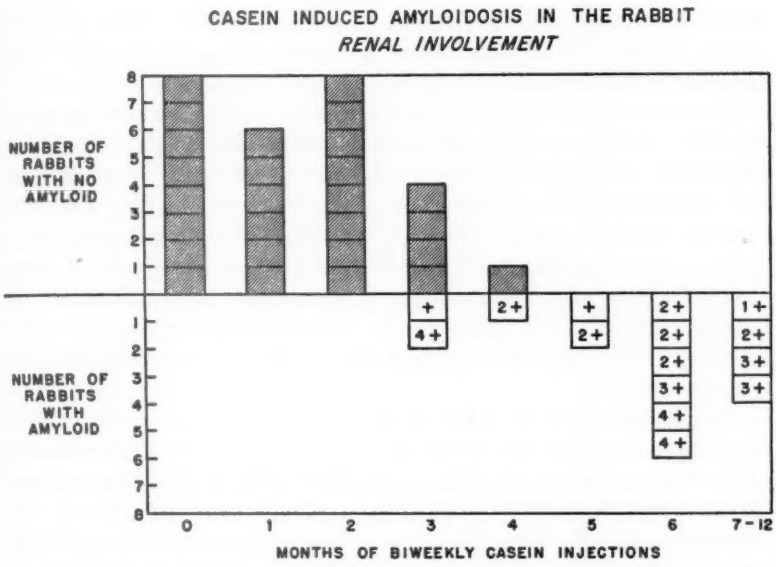
Hepatic amyloidosis (Text-fig. 3) was not marked; the replacement never exceeded 50 per cent of the organ. It was observed only once in rabbits receiving casein for 3 months or less, and was seen in only 5 of 14 animals receiving casein for longer periods. The livers with amyloidosis were also more firm, dark and rubbery. Weights of this organ were not recorded routinely. Lymph node involvement was occasionally encountered, but amyloidosis of the heart, skeletal muscle, gastrointestinal tract, or skin was not observed. Endocrine organs were not regularly examined.



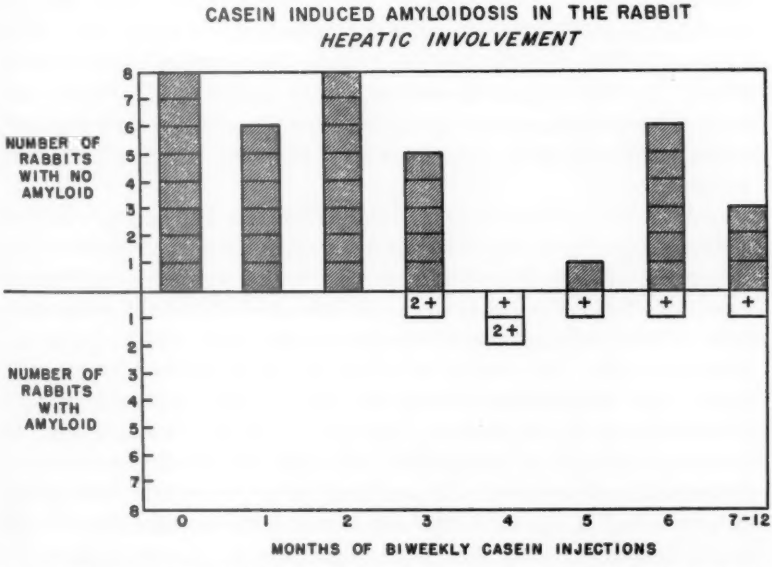
Text-figure 1. The extent of amyloid is graded 0 to 4+, as described in the footnote to Table I.

Histologic Appearance

Spleen. The amyloid initially appeared in the marginal area of the red pulp, i.e., perifollicular region (Figs. 1 and 2). Here one observed a structureless, apparently amorphous, eosinophilic substance which bound Congo red and stained metachromatically with crystal violet. The amyloid accumulated extracellularly in the subendothelial region. An increase in plasma cells and reticuloendothelial cells was frequently seen. Occasionally, the latter contained PAS-positive granules. The heterophils (pseudo-eosinophils in the rabbit) were also increased in



Text-figure 2. The extent of amyloid is graded o to 4+, as described in the footnote to Table I.



Text-figure 3. The extent of amyloid is graded o to 4+, as described in the footnote to Table I.

numbers after one to two months of casein injections. The granules of the latter cells in both treated and control animals bound Congo red, and were distinct from the PAS-positive granules.

Occasionally, in the early stages of the process, isolated nodular deposits in the red pulp or small intrafollicular deposits could be observed. With time and increasing numbers of casein injections, the amyloid appeared to replace most of the red pulp, sparing only trabeculas, portions of the lymphoid follicles, and the capsule (Figs. 3 and 4). In several animals one could observe a small focus of more intensely eosinophilic substance indistinguishable from "fibrinoid" in the red pulp (Fig. 3). This focus bound Congo red weakly, was orthochromatic with crystal violet and stained dark yellow with the van Gieson stain.

In the animals with the most advanced lesions, there was almost total obliteration of the splenic architecture to a point where the sections were no longer recognizable as spleen. The red pulp was replaced by a relatively acellular, amorphous, glassy material. During the development of the amyloidosis, no evidence of simultaneous reabsorption was noted. In only one section was a giant cell observed.

The amyloid substance exhibited similar staining characteristics in all stages of the disease. It was eosinophilic, bound Congo red, and was metachromatic with crystal violet. It stained olive yellow with the van Gieson and light purple with the periodic acid-Schiff stains. Fluorescence microscopy demonstrated a faint blue-green autofluorescence at 365 mμ and a pink fluorescence after staining with Congo red. Polarization microscopy showed a faint birefringence of the unstained, formalin-fixed sections. Staining with Congo red intensified the birefringence.

Kidney. The earliest lesions of amyloidosis in the kidney occurred in the glomerular tufts, where hyaline deposits of eosinophilic substance were found. The distribution of lesions among the glomeruli was fairly even. Further deposition of amyloid within the glomerular tufts resulted in their almost complete replacement (Figs. 5 and 6). The final stage was one of sclerosis and obliteration of glomeruli. Under high magnification, the glomerular deposits appeared in close relationship to the capillary endothelium. Accumulations of amyloid were seen between the endothelial cells and the basement membrane. Occasionally, endothelial cells appeared to be completely surrounded by amyloid (Fig. 6). The capillary lumen in advanced cases was all but obliterated.

Simultaneously with the evolution of the glomerular lesion, similar but less extensive accumulations of amyloid occurred in the interstitial

tissues of the kidney. Most commonly the medullary connective tissue harbored discrete deposits of amyloid, although occasionally subcapsular or nodular deposits could be found in the cortex. The lesion appeared to be most severe in the region of the corticomedullary junction (Fig. 7). Rarely was the interstitial deposit as marked as that in the glomeruli. High-power views of the medullary deposits also showed a close relationship to capillary basement membranes and interstitial connective tissue elements. The staining characteristics of the renal deposits were identical with those in the spleen.

With the PAS stain, the capsular as well as the glomerular basement membranes appeared thickened. Occasionally, within Bowman's space, "crescents" were observed. These were eosinophilic, and orthochromatic when stained with crystal violet; they did not bind Congo red, and appeared yellow with the van Gieson stain. Late in the disease, colloid casts were often present in the tubules. The casts, too, were eosinophilic and did not bind Congo red. With one exception, they were orthochromatic when stained with crystal violet. They usually were stained bright violet with PAS.

Liver. In the series of 34 rabbits treated with casein, only 6 showed hepatic deposit of amyloid, and usually this was not marked. The distribution was periportal in 2 cases, and more diffuse in the remaining 4 (Fig. 8). Two of the latter had a tendency to more marked involvement of the centrilobular region. The amyloid appeared to accumulate between the parenchymal and the Kupffer cells. With progressive deposition, the parenchymal cells were obscured and were replaced by nodular masses of amyloid. The staining characteristics of the amyloid in the liver were identical with those in the spleen and kidney.

Discussion

The observations reported are of interest in two respects. First, they illustrate the histologic sequence in the development of amyloidosis, and second, they provide an opportunity to evaluate the usefulness of various staining and optical methods in the detection of the substance and to learn more of its nature.

Histologic evidence of abnormal activity in the spleen was apparent during the course of casein injections, even before the first appearance of amyloid. Reticuloendothelial proliferation was noted in the marginal zone of the red pulp by the end of one month. At that time increased numbers of plasma cells and heterophils were observed. In some instances PAS-positive, and in rare cases, pyroninophilic substance was found in the reticuloendothelial cells. None of these features, however, were consistently observed in all animals at the end

of one or two months' time, and they varied greatly in magnitude.

The relation of any one of the observations to the pathogenesis of amyloid is uncertain at present. Although plasma cell proliferation is often associated with antibody formation, there are conflicting reports in the literature as to the role immunologic phenomena play in the genesis of amyloid.[11,12] The increase in the number of heterophils noted was far more impressive than the increase in plasma cells. The fact that the granules of the heterophils (pseudo-eosinophils) bound Congo red was considered to be a nonspecific feature since the heterophils of untreated controls exhibited an identical staining reaction.

It was of interest that the greatest and earliest increase of heterophils and plasma cells, and, indeed, the earliest appearance of amyloid, was in the marginal zone of the red pulp. This region has been described as functionally transitional between red and white pulp. It has been said to be a very active part of the spleen, and perhaps the portion controlling blood cell sequestration and other splenic adaptive functions.[13,14]

The presence of PAS-positive and pyroninophilic substances in the spleen has been noted and extensively discussed by Teilum.[15] He regarded them to be indicative of glycoprotein synthesis. This assumption is supported by the fact that the animals, by the end of one month, also demonstrated marked increase in serum hexosamine concentration, indicating increased concentration of serum glycoproteins.[9] On the other hand, in the present investigation, the amount of PAS and pyroninophilic material was far less than that described by Teilum. Some of these differences may be accounted for by differences in histologic technique. However, the evidence that these histologic features *per se* denote abnormal glycoprotein synthesis would seem to be circumstantial. The conclusion that simply because the accumulation of PAS-positive and pyroninophilic material precedes the development of amyloidosis, the former substance is a precursor of the latter does not appear to be justified. The nonspecific nature of the staining reactions and the lack of understanding of their chemical nature make it difficult to appreciate their basic significance.

Histologic evidence of renal amyloidosis was first seen in one rabbit after 3 months of injections, but in most animals it was delayed until the fourth or fifth month. In Richter's[16] studies of amyloidosis induced in rabbits by sodium ribonucleate, the renal lesions also occurred much later than in the spleen. Colloid casts were present in 11 of 15 rabbits with kidney amyloidosis and in 2 of the animals with no renal amyloid. In all instances the casts were eosinophilic, orthochromatic,

strongly PAS positive, and failed to stain with Congo red. These reactions suggest that these proteins differ from amyloid.

Hepatic involvement in this series was minimal. When small accumulations of amyloid did occur, they were localized between Kupffer and parenchymal cells. These observations are similar to those previously reported. The overall distribution in casein-induced amyloidosis of rabbits is, therefore, parenchymal—resembling the so-called "secondary" amyloidosis in human subjects.

The rabbits with experimental amyloidosis provide an excellent opportunity for investigation of the histologic characteristics of this substance in different stages of its development. The appearance of the amyloid was identical from organ to organ and did not vary with duration. The deposit in the spleens of animals sacrificed at 2 to 3 months stained identically with that in those sacrificed at 6 to 8 months. It had similar characteristics in all organs no matter what the time of sacrifice.

Evaluation of Histologic Techniques

Hematoxylin and Eosin. Apart from its obvious use in conventional tissue evaluation, this stain was often sufficiently distinctive to make one suspect amyloid strongly in moderately and severely affected organs. Its use alone, however, was never felt to be conclusive in view of the possibility of confusion of amyloid with other extracellular eosinophilic deposits of different nature. In addition, minimal amounts of amyloid could be overlooked easily.

Congo Red. The use of this dye in the histologic demonstration of amyloid is classic. Nonetheless, it proved to have certain disadvantages in our hands. Unless the sections were carefully decolorized, dense collagenous tissue often bound considerable amounts of the dye. Moreover, the faintness of color precluded its use in fully appraising minimal depositions. As will be described subsequently, however, fluorescence and polarization studies after Congo red staining were more significant.

Metachromatic Dyes. Although metachromatic dyes have been known since 1875, they are only now being characterized.[17] Metachromasia is a subject of controversy because of the complex nature and often impure composition of the dyes involved. However, as a tool in the diagnosis of amyloidosis, it has proved to be of great value. In the present investigation, methyl violet (color index, 680) was first used to test for metachromasia. However, because of occasional variations in staining and the impure composition of this dye

(this has been corroborated by Dr. Børge Larsen, using paper chromatography), crystal violet (color index, 681) was then utilized. It, too, was shown by chromatograms to contain two distinct spots, but despite this, results were technically reproducible. The metachromasia of amyloid was a most useful property in locating early and minimal deposits. In the spleen, rare areas of "fibrinoid" stained orthochromatically. These had a denser, coarser structure which was quite distinguishable from the glassy appearance of amyloid. In addition, these lesions did not bind Congo red.

Van Gieson Stain. In 1889, van Gieson described a dye useful in staining the connective tissue of peripheral nerves.[18] Since then, the value of this dye in differentiating collagen (which stains red) from other connective tissue depositions has become well known. Although in the present investigation the van Gieson stain did not serve to establish the diagnosis of amyloid in any animal, it was useful in differentiating amyloid from collagen. Amyloid in the rabbit stained yellow with this dye and had less of the khaki or orange tint that has been observed in human amyloidosis. The staining quality was also consistent with the observation that amyloid does not contain a significant amount of hydroxyproline, and thus supports the inference that amyloid is not primarily collagenous in nature.[19]

Periodic Acid-Schiff (PAS) Reaction. Although amyloid stained in weakly positive manner with PAS, the use of this dye did not aid in the detection of this substance.[20] During the development of the amyloid, PAS-positive granules were occasionally observed in the reticuloendothelial cells of the spleen. These were not necessarily related to the evolution of the disease.

Pyronin Methyl Green Stain. The tissues of 2 normal and 12 casein-treated rabbits containing varying degrees of amyloidosis were stained with pyronin methyl green.[15] Occasional pyroninophilic cells were observed, but the phenomenon was not striking. It bore no clearcut chronologic or qualitative relationship to the degree of amyloidosis.

Fluorescence Studies. Unstained, formalin-fixed sections, 5 μ in thickness, demonstrated that amyloid substance was autofluorescent when viewed at a wave length of 365 mμ. This did not, however, enable it to be clearly distinguished from other tissue components. Following Congo red staining, however, amyloid exhibited a pink fluorescence. This was distinctive and sharply localized to the amyloid in the well decolorized specimen. When sections were thick, showed folds, or the dye was not adequately decolorized, false positive dye fixation was frequently observed. However, the technique of Congo red staining

and fluorescence microscopy, when carried out meticulously, was useful in delineating small accumulations of amyloid.

Polarization Microscopy. Examination of unstained, formalin-fixed sections demonstrated that amyloid was very weakly birefringent with respect to the long axis of the deposit under observation. As observed by Missmahl and Hartwig,[21,22] the birefringence increased markedly after Congo red staining. In contrast to collagen, which is white on visualization in the polarizing microscope, the unstained and Congo red stained amyloid had a pale green hue. As in the case of collagen, however, the birefringence was positive with respect to the long axis of the deposit. This anisotropy suggests that amyloid may have an orderly intrinsic molecular arrangement accentuated by Congo red binding, and is not completely amorphous in nature.

Missmahl believed that amyloid contained collagen fibers which accounted for its behavior in polarized light. On the other hand, it is possible that its behavior is due to an intrinsic orientation of micelles that compose the amyloid itself. The chemical analyses previously mentioned,[19] however, would seem to indicate that collagen was not a significant part of amyloid. Since birefringence in the present investigation was positive with respect to the long axis of the deposit, it would appear that the submicroscopic units that make it up are oriented parallel to its long axis. More detailed studies of the fine structure of amyloid by electron microscopic methods have been carried out and are the subject of other reports.[23-25]

Summary and Conclusions

1. Amyloidosis was induced in 34 rabbits by subcutaneous casein injections twice weekly for periods up to 12 months. Animals were sacrificed at monthly intervals in order to investigate the sequence of alterations and the magnitude of organ involvement. Eight additional rabbits served as controls.

2. All animals which received the injections for 4 or more months exhibited amyloidosis of some degree.

3. The spleen was affected in most animals after 2 months and was invariably laden with amyloid after 3 to 4 months. The kidney was involved progressively only after 4 to 5 months of injections. The liver was uncommonly affected and contained smaller deposits of amyloid.

4. Tissues were stained with hematoxylin and eosin, Congo red, PAS, van Gieson, crystal violet, and pyronin methyl green stains. In addition, unstained and Congo red stained sections were examined for fluorescence and birefringence.

5. The combination of hematoxylin and eosin, crystal violet and van Gieson stain was most useful in detecting amyloid. Examination with ultraviolet light after Congo red staining was useful in detecting minimal deposits.

6. Polarization studies demonstrated positive birefringence in amyloid, suggesting that it may have an organized molecular rather than an amorphous structure.

REFERENCES

1. Hjärre, A. Über das Vorkommen der Amyloiddegeneration bei Tieren. *Acta path. et microbiol. scandinav.*, 1933, Suppl. **16**, 132–162.
2. Giles, R. B., Jr., and Calkins, E. Studies of the composition of secondary amyloid. *J. Clin. Invest.*, 1955, **34**, 1476–1482.
3. Dick, G. F., and Leiter, L. Some factors in the development, localization and reabsorption of experimental amyloidosis in the rabbit. *Am. J. Path.*, 1941, **17**, 741–754.
4. Gellhorn, A.; van Dyke, H. B.; Pyles, W. J., and Tupikova, N. A. Amyloidosis in hamsters with leishmaniasis. *Proc. Soc. Exper. Biol. & Med.*, 1946, **61**, 25–30.
5. Pirani, C. L.; Bly, C. G.; Sutherland, K., and Chereso, F. Experimental amyloidosis in the guinea pig. *Science*, 1949, **110**, 145–146.
6. Kuczynski, M. H. Edwin Goldmann's Untersuchungen über celluläre Vorgänge im Gefolge des Verdauungsprozesses auf Grund nachgelassener Präparate dargestellt und durch neue Versuche ergänzt. *Virchows Arch. path. Anat.*, 1922, **239**, 185–302.
7. Jaffé, R. H. Amyloidosis produced by injections of proteins. *Arch. Path.*, 1926, **1**, 25–36.
8. Dunn, T. B. Relationship of amyloid infiltration and renal disease in mice. *J. Nat. Cancer Inst.*, 1944, **5**, 17–28.
9. Giles, R. B., Jr., and Calkins, E. The relationship of serum hexosamine, globulins, and antibodies to experimental amyloidosis. *J. Clin. Invest.*, 1958, **37**, 846–857.
10. Calkins, E.; Cohen, A. S., and Schubart, A. Studies on experimental amyloidosis. II. The relation of serum changes and method of casein administration to the production of amyloidosis in rabbits. (In preparation.)
11. Vazquez, J. J., and Dixon, F. J. Immunohistochemical analysis of amyloid by the fluorescence technique. *J. Exper. Med.*, 1956, **104**, 727–736.
12. Calkins, E.; Cohen, A. S., and Gitlin, D. Immunochemical determinations of gamma globulin content of amyloid. (Abstract) *Fed. Proc.*, 1958, **17**, 431.
13. Snook, T. A comparative study of the vascular arrangements in mammalian spleens. *Am. J. Anat.*, 1950, **87**, 31–77.
14. Weiss, L. Aspects of the reticuloendothelial system studied with the light microscope and the electron microscope. *Ann. N.Y. Acad. Sc.*, 1958, **73**, 131–138.
15. Teilum, G. Periodic acid-Schiff-positive reticulo-endothelial cells producing glycoprotein. Functional significance during formation of amyloid. *Am. J. Path.*, 1956, **32**, 945–959.
16. Richter, G. W. The resorption of amyloid under experimental conditions. *Am. J. Path.*, 1954, **30**, 239–261.

17. Schubert, M., and Hamerman, D. Metachromasia; chemical theory and histochemical use. *J. Histochem.*, 1956, **4**, 159–189.

18. Van Gieson, I. Laboratory notes of technical methods for the nervous system. *New York Med. J.*, 1889, **50**, 57–60.

19. Calkins, E., and Cohen, A. S. Chemical composition of amyloid. *J. Clin. Invest.*, 1958, **37**, 882–883.

20. McManus, J. F. A. The periodic acid routine applied to the kidney. *Am. J. Path.*, 1948, **24**, 643–653.

21. Missmahl, H. P., and Hartwig, M. Polarisationsoptische Untersuchungen an der Amyloidsubstanz. *Virchows Arch. path. Anat.*, 1953, **324**, 489–508.

22. Missmahl, H. P. Polarisationsoptischer Beitrag zur Kongorotfärbung des Amyloid. *Ztschr. wiss. Mikr.*, 1957, **63**, 133–139.

23. Cohen, A. S.; Weiss, L., and Calkins, E. A study of the fine structure of the spleen in experimental amyloidosis of the rabbit. (Abstract) *Clin. Res.*, 1958, **6**, 237.

24. Cohen, A. S., and Calkins, E. A light and electron microscopic study of human and experimental amyloid disease of the kidney. (Abstract) *Arthritis and Rheumatism*, 1959, **2**, 70–71.

25. Cohen, A. S., and Calkins, E. Electron microscopic observations on a fibrous component in amyloid of diverse origins. *Nature, London*, 1959, **183**, 1202–1203.

The authors express their gratitude to Drs. Walter Bauer, Benjamin Castleman, Jerome Gross and Leon Weiss for encouragement and suggestions, and to Miss Jean MacIntosh and Mr. Orville Rodgers for technical assistance.

[*Illustrations follow*]

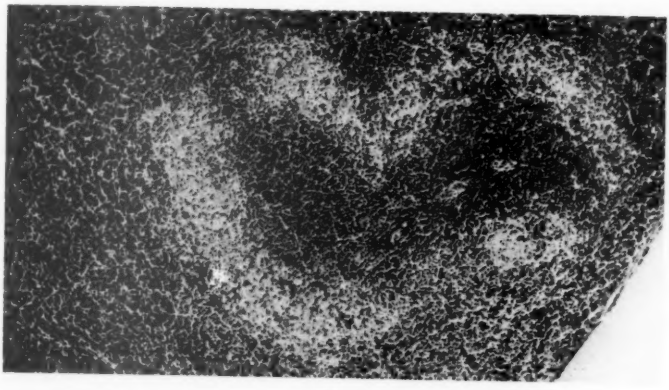
LEGENDS FOR FIGURES

All sections illustrated were stained with hematoxylin and eosin.

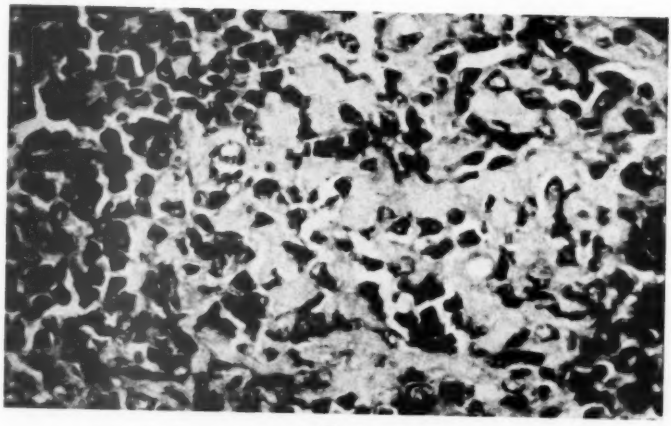
FIG. 1. Spleen of rabbit No. 148 (Table I). Perifollicular distribution of amyloid; an early stage of involvement, classified as 1+. × 80.

FIG. 2. Higher magnification of Figure 1, demonstrating amyloid in the marginal zone of the red pulp. Amyloid appears to be located in the subendothelial region. × 450.

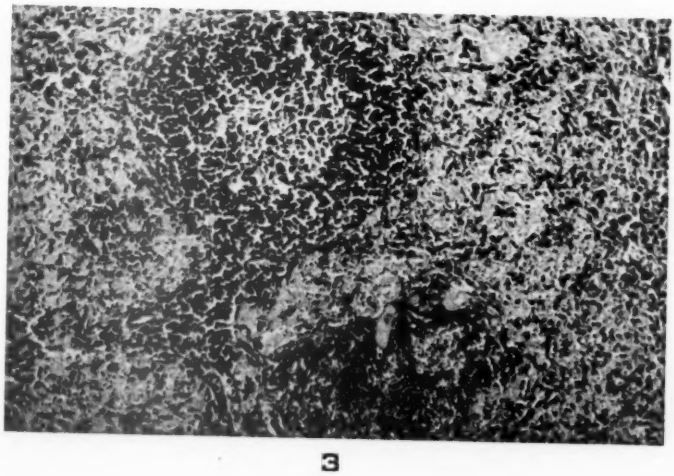
FIG. 3. Spleen of rabbit No. 99 (Table I). Lymphoid follicle, perifollicular amyloid (3+) and darker staining "fibrinoid" (F) amidst the amyloid. × 125.



1



2

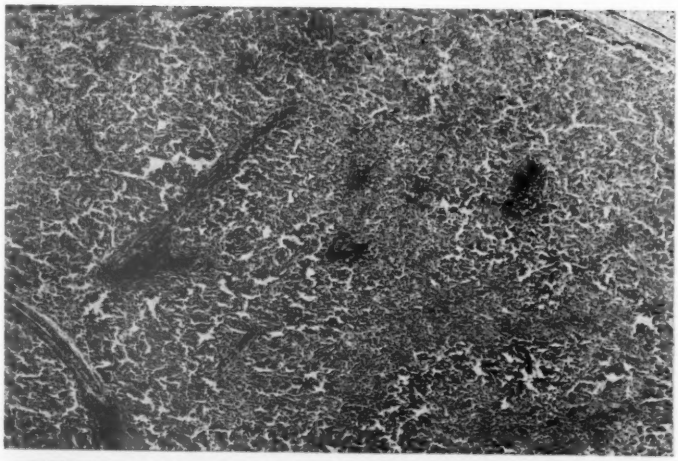


3

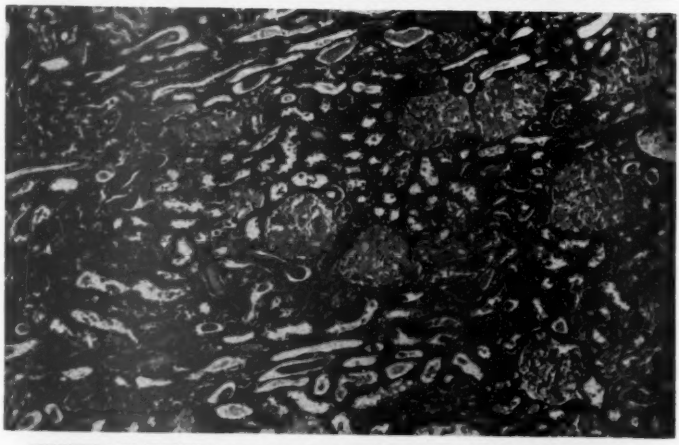
FIG. 4. Spleen of rabbit No. 56, with advanced amyloidosis (4+). Almost total replacement of the organ by amyloid, with only scattered remnants of trabeculas and lymphoid follicles. × 37.

FIG. 5. Kidney of rabbit No. 58. Advanced amyloidosis of renal cortex, showing almost complete replacement of glomeruli with amyloid. Tubular casts are present in the medullary rays. × 80.

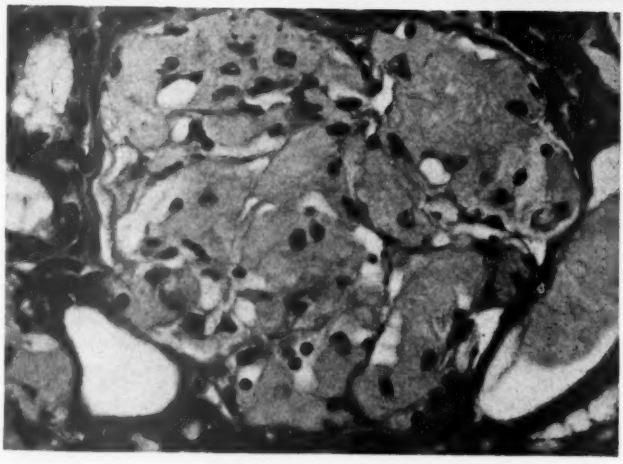
FIG. 6. Kidney of same rabbit. Glomerulus with advanced amyloidosis. Amyloid appears to be localized between the basement membrane and endothelial cells of the glomerulus, with the latter occasionally surrounded by amyloid substance. × 450.



4



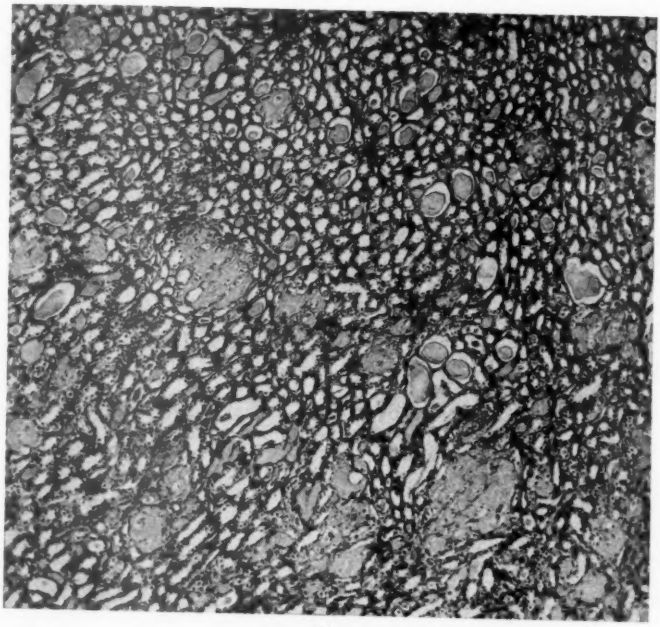
5



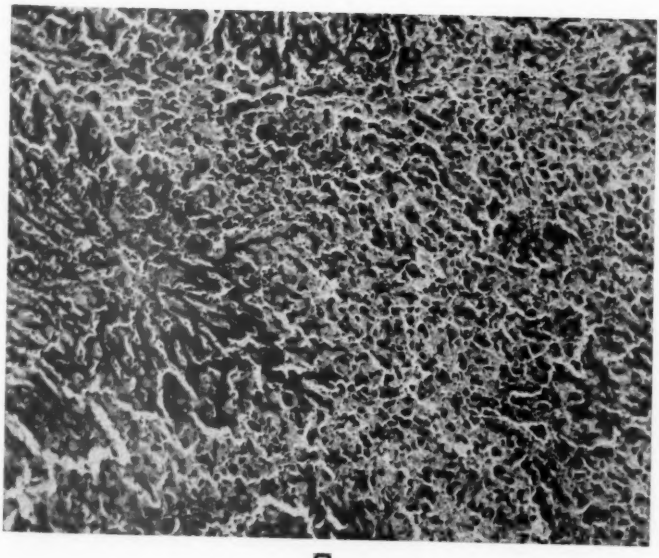
6

FIG. 7. Same rabbit kidney, showing amyloidosis of renal medulla. Involvement is most severe at the corticomedullary junction. Many tubular casts are also present. × 80.

FIG. 8. Liver of rabbit No. 115. Small amounts of amyloid in periportal and centrilobular regions. Parenchymal cell destruction may be seen in areas of amyloid deposition. × 80.



7



8

STAINING METHODS FOR OSMIUM-METHACRYLATE SECTIONS*

BARBARA M. JENNINGS; MARILYN G. FARQUHAR, Ph.D.†, and HENRY D. MOON, M.D.
From the Department of Pathology, University of California School of Medicine, San Francisco, Calif.

In the study of tissues by electron microscopy, it is desirable to have contiguous sections available for examination by light microscopy for purposes of orientation and selection of appropriate fields. Tissues fixed in osmium tetroxide and embedded in methacrylate often provide suboptimal results when stained by the usual methods for light microscopy.[1] The purpose of this investigation was to develop methods of staining these tissues and to determine which histochemical techniques could be applied without modification.

The procedures were used primarily on renal tissues obtained by needle biopsy. Excellent results were obtained with a number of different staining methods; several are illustrated in a color plate recently published.[2] Some of the techniques have been found to be applicable to other tissues, such as eyes, arteries, and developing teeth. Two of the methods yielded good results with the pituitary gland of the rat.

MATERIAL AND METHODS

Of the many groups of dyes tested, the tri-phenyl methane derivatives were the most satisfactory. These dyes included malachite green,‡ light green SF yellowish, fast green FCF, basic fuchsin, acid fuchsin, methyl violet,‡ crystal violet,‡ ethyl green, and aniline blue. Other satisfactory groups were: (a) nitro and azo dyes: picric acid, orange G, ponceau 2R, Biebrich scarlet, Bismarck brown R, Congo red; (b) quinone-imines: thionine, azure A, methylene blue, toluidine blue O,§ gallocyanin, Celestin blue B, safranin O,‡ and azocarmines B and G; (c) xanthenes: pyronin B, eosin Y, ethyl eosin, erythrosin, and phloxine B; (d) phthalocyanin: Alcian blue 8GS; and (e) natural dyes: hematoxylin and cochineal (carmine).

A new staining method was devised employing basic fuchsin, alum, and hematoxylin (FAH). Staining procedures which were modified were the hematoxylin and eosin, Mallory-Heidenhain "azan," Mallory connective tissue, Masson trichrome, colloidal iron stains, and the

* This investigation was aided by grants P-112 and 113 from the American Cancer Society, and by grant A-645 of the National Institutes of Health, United States Public Health Service.
Received for publication, December 22, 1958.
† Present address: The Rockefeller Institute, New York 21, N.Y.
‡ Aqueous mount.
§ Acetone dehydrated.

methyl violet stain for amyloid. Staining methods applied without change were the periodic acid-Schiff, Feulgen, methenamine silver, von Kóssa, gallocyanin-chromalum, Alcian blue, and the following elastic tissue stains[3]: Gomori's aldehyde fuchsin, Weigert's resorcin fuchsin, Unna's orcein, and Verhoeff's iodine-iron-hematoxylin.

Preparation of Tissues

Renal tissue was obtained by needle biopsy[4] and other tissues by open biopsy or at necropsy. Tissues were cut into 1 mm. cubes and fixed for 1½ to 2 hours in 1 per cent osmium tetroxide buffered at pH 7.4 with veronal acetate.[5] Sucrose was added to the solution of osmium tetroxide according to the method of Caulfield.[6] The tissue was dehydrated in graded alcohols, embedded in n-butyl methacrylate containing 3 per cent paste catalyst (1,2-dichlorobenzoyl peroxide), and incubated at 45° C. for 18 to 24 hours. The details of the fixation and embedding procedure may be found elsewhere.[7]

Sections were cut with a Porter-Blum microtome at 1 to 6 μ, depending upon the stain to be employed (see individual staining procedures). Sections were floated onto a water bath containing 20 per cent acetone, mounted on albuminized slides, and air dried. Before staining, sections were passed through xylene to remove methacrylate and then through graded alcohols to water.

Staining Procedures

Fuchsin-Alum-Hematoxylin (FAH). Goodpasture's aniline carbol fuchsin solution, MacCallum's variant[8]:

30% alcohol	100	ml.
Basic fuchsin	0.59	gm.
Aniline	1	ml.
Phenol	1	gm.

Note: This solution is stable and may be reused for several months.

Method: (Sections cut at 1 to 2 μ.)

1. Stain in Goodpasture's solution for 1 minute.
2. Rinse quickly in distilled water.
3. Treat with 5% aqueous ferric ammonium sulfate for 5 minutes.
4. Rinse in distilled water.
5. Stain in Harris' hematoxylin[8] for 15 minutes.
6. Rinse in distilled water.
7. Rinse in tap water, 1 to 2 minutes.
8. Dehydrate quickly in 95% and 100% alcohols, clear in xylene, and mount.

Results: Nuclei, pink or blue-gray; endothelial cytoplasm, grayish rose; basement membrane, rose to purple; connective tissue fibers, blue-gray; hyalin of diabetes (early), bright rose; (late), purple; amyloid, bright rose; calcium, dark blue to black; pituitary acidophil granules, rose.

Hematoxylin and Eosin Variant.

Method: (Sections cut at 1 to 2 μ.)

1. Stain in Harris' hematoxylin for 1 to 2 minutes.

2. Wash in running tap water for 2 minutes.
3. Counterstain in 1% eosin Y in 0.1% aqueous solution of calcium chloride for 5 minutes.
4. Dehydrate quickly in 95% and 100% alcohols, clear in xylene, and mount.

Results: Nuclei, blue; cytoplasm, pink.

Note: 1% phloxine B in 0.1% $CaCl_2$ or 1% erythrosin in 0.01% $CaCl_2$ may be substituted for eosin Y.

Mallory-Heidenhain "Azan" Variant.

Mallory III solution[3]:

Aniline blue	0.5 gm.
Orange G	2 gm.
Acetic acid	8 ml.
Distilled water	100 ml.

Method: (Sections cut at 4 to 6 μ.)

1. Stain in 1% azocarmine B in 1% acetic acid for 4 hours at 50 to 55° C.
2. Rinse in distilled water.
3. Counterstain in Mallory III for 5 minutes (aniline blue in aqueous or oxalic acid solutions, and light green or fast green in acetic acid solution may be substituted.)
4. Rinse in distilled water.
5. Dehydrate in 95% and 100% alcohols, clear in xylene, and mount.

Note: A Mallory connective tissue variant may be employed by substituting azocarmine with 0.5% cold aqueous acid fuchsin for 30 minutes.

Results: Nuclei, red; basement membrane, blue; collagen, blue; glomerular fibrinoid, orange-red.

Masson Trichrome Variant.

Method: (Sections cut at 4 to 6 μ.)

1. Stain in Weigert's acid iron chloride hematoxylin (Lillie's variant[3]) for 10 minutes.
2. Rinse in distilled water.
3. Rinse in tap water for 3 minutes.
4. Stain in ponceau-acid fuchsin[3] for 30 minutes.
5. Rinse quickly in distilled water.
6. Stain in 2% light green SF yellowish in 2% acetic acid for 5 to 10 minutes. (1% fast green FCF in 1% acetic acid for 3 to 5 minutes may be substituted.)
7. Rinse quickly in distilled water.
8. Dehydrate in 95% alcohol (check degree of light green staining), 100% alcohol, clear in xylene, and mount.

Note: Because red tones were diminished by use of phosphomolybdic and phosphotungstic acid mordants (customarily applied in trichrome staining), these were omitted.

Results: Nuclei, dark gray; basement membranes and hyalin of diabetic glomerulosclerosis, green; collagen, green; arteriolar hyalin and fibrinoid, red.

Colloidal Iron Variants.

Method: (Section pituitary at 4 to 6 μ; other tissues at 1 to 2 μ.) Technique of Rinehart and Abul-Haj,[9] with the following modifications after the colloidal iron-ferrocyanide steps:

1. Rinse in distilled water.
2. Treat with 0.5% aqueous periodic acid or sulfurous acid (6 ml. of 10% sodium meta-bisulfite, 5 ml. of 1 N HCl, 100 ml. distilled water) for 5 minutes.
3. Wash in running tap water, 5 minutes.

4. Stain in Grenacher's alum carmine[8] for 40 minutes. (Omit for sections of pituitary.)
5. Rinse in distilled water.
6. Counterstain in 0.5% aqueous acid fuchsin for 15 minutes.
7. Rinse in distilled water.
8. Dehydrate in 95% and 100% alcohols, clear in xylene, and mount in polystyrene resin.[8]

Note: This technique may be applied to formol-paraffin sections if the staining time in acid fuchsin is reduced to ½ minute or less and followed by differentiation in tap water.

Results: Nuclei, blue or rose; cytoplasm of glomerular epithelium, light blue; cytoplasm of glomerular endothelium, pink; connective tissue ground substance, blue; collagen, pink; glomerular hyalin (diabetic), pink; basement membrane, pink; pituitary gland: acidophils, pink; basophils, blue; Golgi apparatus, pink.

Another variant of the colloidal iron technique is the application of the periodic acid-Schiff (PAS) reaction following the colloidal iron-ferrocyanide steps.[10] (Alcohol rinses are replaced with distilled water and the thiosulfate reducing rinse with a 5 minute tap water wash.) After the final sulfurous acid rinse nuclei may be stained with Harris' hematoxylin. The results given above will then be changed to light gray or blue nuclei and dark rose collagen, basement membranes, endothelial cytoplasm, and glomerular hyalin of diabetes.

Periodic Acid-Schiff.

Method: (Sections cut at 4 to 6 μ.) Method of McManus.[11] Counterstain with Harris' hematoxylin and fast green if nuclear and connective tissue staining is desired. Leucofuchsin (Schiff) reagents: cold aqueous preparation of Longley,[12] boiled aqueous preparation of Coleman,[13] or the following alcoholic preparation:

1. Mix together:
   100% alcohol ..... 70 ml.
   Basic fuchsin ..... 0.5 gm.
2. Mix, dissolve, and add to the above solution:
   Hydrochloric acid (conc.) ..... 3 ml.
   Distilled water ..... 30 ml.
   Sodium meta-bisulfite ..... 1 gm.
3. Store in tightly stoppered bottle in dark overnight until solution is amber. Shake with 0.3 gm. activated charcoal for 2 minutes and filter through Whatman #2 paper. Store in dark at 0 to 5° C. When the solution has further decolorized to pale buff, it is ready for use.

Note: 1. Two alternate oxidants for the Schiff reaction (1% potassium permanganate and 4% chromic acid) were tested, but the staining was less intense.
2. The aqueous leucofuchsin reagents listed above can be used for the Feulgen reaction[14] after hydrolyzing tissues for 30 minutes in 1 N HCl at 60° C.

Results: Positive PAS reaction in basement membrane, hyalin, "fibrinoid," and glycogen.

Methyl Violet Variant for Amyloid.

Method: (Sections cut at 4 to 6 μ.)

1. Stain in 1% aqueous methyl violet 2B (color index #680) for 5 minutes.
2. Rinse in distilled water (to redden amyloid).
3. Decolorize connective tissue quickly (to light blue) with 80% alcohol (see note below).
4. Stop decolorization with distilled water rinse.
5. Drain and mount in glycerol gelatin (Kaiser-Mallory).[8]

Note: Because the customary acetic acid differentiating rinse (Mallory, Lillie)[3] decolorized amyloid, it was replaced by alcohol.
Results: Amyloid, dark rose; other glomerular structures, light rose to light blue.

Discussion

Standard histologic staining methods were altered for application to osmium-methacrylate tissues by increasing staining times, omitting or varying some mordanting and differentiating rinses, and substituting dyes where the original did not stain. Tissue sections were cut at 1 to 6 μ, depending upon the staining procedure to be used.

Some sections mounted in Permount® (Fisher Scientific Company, New York) faded rapidly. Since Permount is excellent for preserving most tissue stains, fading is apparently peculiar to osmium-fixed, methacrylate-embedded tissues. An acrylic resin, Krylon ("Crystal Clear" #1303, Krylon, Inc., Norristown, Pennsylvania) was tried as a mounting medium without the use of cover slips,[15] and there was less fading. The refractive index of acrylic resins (1.49) was not high enough to give a sharp image under high dry lens; however, with immersion oil and the 97x lens, definition was far greater than in sections mounted in Permount.

The fuchsin-alum-hematoxylin (FAH) method was simple and rapid and gave constant results. Aniline carbol fuchsin, which demonstrated hyalin, amyloid, and pituitary acidophils in this technique, was selected because it could be counterstained and dehydrated without being decolorized.

The connective tissue stains which were adapted were Mallory-Heidenhain "azan," Masson trichrome, and Mallory connective tissue. Elimination of decolorizing and mordanting acids and careful control of staining times minimized the diffuse and variable staining reactions experienced by Houck and Dempsey.[1]

McManus' periodic acid-Schiff and Gomori's methenamine silver techniques were applied without variation. Both methods demonstrated hyalin, fibrinoid, basement membrane of renal glomeruli and tubules, and Bruch's membrane of the eye. In accordance with the experience of others,[16,17] methenamine silver provided greater contrast than PAS so that thinner sections (1 to 2 μ) could be examined; these provided better cytologic detail. The use of periodic acid as an oxidant in the methenamine silver method gave greater basement membrane contrast, whereas chromic acid oxidation provided nuclear detail. It has also been possible to show glycogen with the PAS reaction and reticulum fibers by using Wilder's ammoniacal silver nitrate in osmium-methacrylate tissues.[1]

A variant of the hematoxylin and eosin technique was developed by the addition of 0.01 to 0.1 per cent calcium chloride to the fluorans (eosins). This procedure intensified the staining reaction of the fluorans[3,18] and prevented their decolorization in dehydrating alcohols.

Nuclear chromatin was stained by the Feulgen and gallocyanin-chromalum[19] methods. A modification of the latter technique, using phloxine B as a counterstain, was developed by Runge, Vernier and Hartmann[20] for use on osmium-methacrylate tissues. However, this method has the disadvantage of requiring a staining time of 24 to 48 hours.

Nuclei were easily stained by methyl green-pyronin methods, but results were too capricious to make them valuable for histochemical determinations. A rapid nuclear and cytoplasmic stain was possible with chloroform extracted methyl green[18] counterstained with 0.5 per cent aqueous acid fuchsin; however, results varied with slight changes in staining times and rapidity of alcoholic dehydration.

Colloidal iron and Alcian blue[21] stains produced intense colors but did not appear specific for acid mucopolysaccharides. The colloidal iron variant could be used for staining of both osmium-methacrylate and formalin-paraffin tissues. Up to the present this has been the only procedure which has provided differential staining of the cells of the anterior pituitary following osmium-methacrylate treatment.

With the methyl violet and FAH stains amyloid was demonstrated and its presence confirmed by examining paraffin sections from the same specimens stained by standard techniques for amyloid.

Summary

A number of staining procedures were successfully applied to osmium-fixed, methacrylate-embedded tissues. A new technique employing fuchsin-alum-hematoxylin (FAH) was devised. Several of the histochemical reactions could be utilized without modification. It was necessary to vary a number of standard procedures because of the difficulty in counterstaining osmium-methacrylate tissues. By changes in mordants and decolorizers and by the substitution of dyes, variants of standard techniques gave results which reproduced those obtained with formalin-fixed, paraffin-embedded sections.

By the use of these techniques it was possible to obtain excellent material for examination by light microscopy. Moreover, contiguous sections were available for investigation by both electron and light microscopy.

References

1. Houck, C. E., and Dempsey, E. W. Cytological staining procedures applicable to methacrylate-embedded tissues. *Stain Technol.*, 1954, **29**, 207–211.

2. Farquhar, M. G.; Hopper, J., Jr., and Moon, H. D. Diabetic glomerulosclerosis; electron and light microscopic studies. *Am. J. Path.*, 1959, **35**, 721–753.
3. Lillie, R. D. Histopathologic Technic and Practical Histochemistry. The Blakiston Co., Inc., New York, 1954, 501 pp.
4. Lusted, L. B.; Mortimore, G. E., and Hopper, J., Jr. Needle renal biopsy under image amplifier control. *Am. J. Roentgenol.*, 1956, **75**, 953–955.
5. Palade, G. E. A study of fixation for electron microscopy. *J. Exper. Med.*, 1952, **95**, 285–298.
6. Caulfield, J. B. Effects of varying the vehicle for OsO_4 in tissue fixation. *J. Biophys. & Biochem. Cytol.*, 1957, **3**, 827–830.
7. Farquhar, M. G. Preparation of ultrathin tissue sections for electron microscopy. Review and compilation of procedures. *Lab. Invest.*, 1956, **5**, 317–337.
8. MacCallum, W. G. A stain for influenza bacilli in tissues. A combination of Goodpasture's and Weigert's stains. *J. A. M. A.*, 1919, **72**, 193.
9. Rinehart, J. F., and Abul-Haj, S. K. An improved method for histologic demonstration of acid mucopolysaccharides in tissues. *A. M. A. Arch. Path.*, 1951, **52**, 189–194.
10. Ritter, H. B., and Oleson, J. J. Combined histochemical staining of acid polysaccharides and 1,2 glycol groupings in paraffin sections of rat tissues. *Am. J. Path.*, 1950, **26**, 639–645.
11. McManus, J. F. A. The periodic acid routine applied to the kidney. *Am. J. Path.*, 1948, **24**, 643–653.
12. Longley, J. B. Effectiveness of Schiff variants in the periodic-Schiff and Feulgen nucleal technics. *Stain Technol.*, 1952, **27**, 161–169.
13. Coleman, L. C. Preparation of leuco basic fuchsin for use in the Feulgen reaction. In: Notes on Technic. *Stain Technol.*, 1938, **13**, 123–124.
14. Feulgen, R., and Rossenbeck, H. Mikroskopisch-chemischer Nachweis einer Nucleinsäure von Typus der Thymonucleinsäure und die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Präparaten. *Ztchr. phys. Chem.*, 1924, **135**, 203–248.
15. Vroman, L. Acrylic spray as a substitute for coverslips. *Am. J. Clin. Path.*, 1953, **23**, 516.
16. Gomori, G. Microscopic Histochemistry: Principles and Practice. University of Chicago Press, Chicago, 1952, 273 pp.
17. Jones, D. B. Nephrotic glomerulonephritis. *Am. J. Path.*, 1957, **33**, 313–329.
18. Conn, H. J. Biological Stains. A Handbook on the Nature and Uses of the Dyes Employed in the Biological Laboratory. Biotech Publications, Geneva, N.Y., 1953, ed. 6, 367 pp.
19. Einarson, L. On the theory of gallocyanin-chromalum staining and its application for quantitative estimation of basophilia. A selective staining of exquisite progressivity. *Acta path. et microbiol. scandinav.*, 1951, **28**, 82–102.
20. Runge, W. J.; Vernier, R. L., and Hartmann, J. F. A standing method for sections of osmium-fixed, methacrylate-embedded tissue. *J. Biophys. & Biochem. Cytol.*, 1958, **4**, 327–328.
21. Steedman, H. F. Alcian blue 8GS: a new stain for mucin. *Quart. J. Micr. Sci.*, 1950, **91**, 477–479.

A STUDY OF THE PATHOGENESIS OF FAT EMBOLISM BASED ON HUMAN NECROPSY MATERIAL AND ANIMAL EXPERIMENTS*

V. S. LeQuire, M.D.; J. L. Shapiro, M.D.; C. B. LeQuire, M.D.; C. A. Cobb, Jr., M.D., and W. F. Fleet, Jr., M.D.

From the Departments of Anatomy, Pathology, and Surgery, Vanderbilt University School of Medicine, Nashville, Tenn.

The frequency of fat embolism as a complication of trauma and its recognition as a clinical entity have been recently emphasized by Peltier.[1] However, controversy persists as to its importance *per se* as a cause of death and as to the origin of the fat which comprises the emboli. The concept that fat embolism occurs as a result of the release of depot fat from traumatized tissues, particularly long bone fractures, has its strongest support in the occasional demonstration of myeloid tissue within pulmonary vessels.[2,3] The contrary view, occasionally voiced, that embolic fat is derived from the circulating blood lipids has had little direct evidence in its support.

Lehman and Moore in 1927[4] brought into question the origin of embolic fat from fat depots by their calculation that the amount of fat contained within a femur was insufficient to cause death by embolization. These data were derived from experiments with normal animals and would not necessarily hold in the case of traumatized animals. This point was emphasized by Whiteley[5] who demonstrated that with associated trauma, death occurred with lesser amounts of injected fat. As a result of their work, Lehman and Moore[4] suggested that products of tissue decomposition, as well as fat solvents, were capable of breaking the colloidal suspension of plasma lipids and that these lipids might be the source of the fat aggregates within vessels. Support for this view has been slow in evolving, but recently the work of Davis and Musselman,[6] as well as that of Johnson and Svanborg[7] and others, has lent support to Lehman's original suggestion that the fat observed in fat embolism was derived from the circulating lipids as well as from marrow and other fat depots.

The observations we have made in cases of fat embolism at necropsy and subsequently in experimental animals are pertinent and lead us to suggest an explanation for fat embolism which satisfies many of the questions which arise concerning pathogenesis when this phenomenon is considered critically. Though the term "fat embolism" denotes

* This investigation was supported by United States Public Health Service Grant (1570), a Senior Researth Fellowship (SF 54) from the Public Health Service, The Middle Tennessee Heart Association, and the Eli Lilly Foundation.

Received for publication, January 5, 1959.

origin elsewhere and transport and impaction in the blood vessels, this concept may be, at least in part, fallacious. The term fat embolism is retained because of common usage and risk of confusion if another designation were introduced.

Necropsy Observations

The present report concerns a series of 9 patients in whom intravascular fat in moderate to large amounts was demonstrated in the lungs. Fat within the vessels of the cerebrum, kidneys and various other organs was present in cases where fat stains were performed on these tissues, but the present investigation was largely confined to examination of the lungs.

Of the 9 patients included, 8 had sustained severe trauma to the skeletal system and soft tissues, with death ensuing at various intervals following the accident. It is noteworthy that 2 patients, dying only 2 to 3 hours after injury, exhibited fat embolism of moderate degree in the pulmonary vessels. Shock was evident in most of the patients; in some it was impossible to determine from the clinical data available whether or not there was an episode of vascular collapse. Another case of fat embolism gleaned from the necropsy files occurred in association with bilateral renal cortical necrosis, with onset immediately after the termination of pregnancy.

In each instance, intravascular fat was easily demonstrated within the small vessels of the lung. Frozen sections stained by Sudan IV and Nile blue sulfate were routinely used. The latter stain, though not specific for fat, is water soluble and gave a more distinct localization and definition of the lipid than did the more commonly used acetone-alcohol-soluble Sudan IV stain. In examining the distribution of fat within the pulmonary vessels, it was observed that masses of crystals were incorporated within the matrix of the fat. The crystalline material did not stain by the above methods; it occurred in sheaf-like arrangements and was acicular in form (Figs. 1 to 6). Once observed, these crystalline aggregates were constantly found without difficulty in the other cases. Examination by means of crossed Nicol prisms facilitated the search for and study of this material since birefringence is a distinguishing feature of the crystals.

They were observed in unfixed tissue obtained at necropsy, but whether or not they appeared as breakdown products of a more complex precursor cannot be said.[8] The birefringence of the crystals disappeared when the sections were heated to 60° C. and returned on cooling. During this procedure, the acicular form did not change. It

has been reported that the birefringence of cholesterol esters disappears at about 60°.[9]

Further investigation of the crystals had to do with their identification, which resolved itself into a study of solubility and histochemical reactions. The crystals were soluble in such fat solvents as alcohol, alcohol-ether mixtures, acetone and xylene. The crystals, as well as the sudanophilic material, gave a positive reaction to the Schultz modification of the Liebermann-Burchardt sterol reaction. Since this test is quite specific but relatively insensitive for cholesterol or cholesterol esters, the indication is that these substances were present in amounts exceeding 10 to 30 per cent of the material comprising the embolic fat.[10] Depot fat, on the other hand, contains less than one per cent cholesterol[11] and does not give a positive reaction to the Schultz test. Birefringence persisted after exposure of the tissues to digitonin followed by an alcohol-ether mixture but was apparently reduced in amount. The Schultz-positive material, as represented by the sudanophilic embolic fat, had disappeared also. This method, described by Feigin,[12] presumably distinguishes between alcohol-ether soluble cholesterol esters and the insoluble, digitonin-precipitable free cholesterol. The results observed indicated that both free and esterified cholesterol were represented in the embolic fat and that most of the Schultz-positive material was present in the non-birefringent embolic fat. It is worthy of note that the birefringent material, noticed initially in the lung vessels, was found in other sites of fat embolism as well (Figs. 9 and 10).

Other microscopic features of note in the lung sections showing fat embolism were (protein-rich) edema and exudation of leukocytes, suggesting an early inflammatory reaction.

In light of the necropsy observations cited, and the report that fat embolism is found in decompression to altitude and caisson disease,[13] the following animal experiments were performed.

Material and Methods

Nineteen rabbits of mixed breed, 8 to 12 weeks of age, weighing 2 to 3 kg. were subjected to hypoxia by decompression in the range of 410 to 340 mm. of mercury. The animals were fed a diet of Purina rabbit pellets before and during the experiments.

Eight animals survived the procedure for 24 to 96 hours; 11 animals died after 1 to 12 hours in the decompression chamber and were excluded from the lipid studies. However, the histologic features on the latter group were of great interest.

Serum samples for lipid analysis were obtained by cardiac puncture 4 to 7 days prior to decompression and immediately after decompression. Following each cardiac puncture, an intravenous injection of heparin (furnished by the Abbott Laboratories), 2.5 mg. per kg. of body weight, was given, and plasma samples were obtained 5 minutes after injection. These, as well as aliquots of the pre-heparin samples, were diluted 1 to 20 with a fat emulsion substrate consisting of 0.03 ml. of a 30 per cent cottonseed oil emulsion (furnished by Dr. H. C. Meng, Department of Physiology, Vanderbilt University) in 100 ml. of phosphate buffer, pH 6.6. Incubation at 37° C. was carried out for 6 hours. The injection of heparin is followed by the appearance in the blood of an enzyme which will catalyze the hydrolysis of triglycerides *in vivo* or *in vitro* with a resulting clearing of the turbidity of lipemic plasma or fat emulsion substrates.[14] The heparin-induced clearing activity of the rabbits' serums was measured by the decrease in optical density which occurred following incubation of the serum with the fat emulsion substrate. For this purpose a Beckman Model B Spectrophotometer was used at 540 mμ as in experiments previously reported.[15]

The surviving animals were killed with intravenous Nembutal,® and tissues from all animals were removed and fixed in 10 per cent formalin. Frozen sections were prepared and examined by the same procedures used for human tissues.

Serum lipids were determined by the method of Bloor[16] for total lipids; the figures given represent total fatty acids and cholesterol. Total cholesterol was also determined by the method of Bloor,[17] and lipid phosphorus was determined by the method of Youngburg[18] which employs the colorimetric reaction of Fiske and Subbarow.[19]

Results of Animal Experiments

Rabbits Surviving Decompression Hypoxia

Table I summarizes the response of 8 rabbits which survived decompression for 24 to 96 hours. The heparin-induced clearing activity was consistently reduced following decompression, and the degree of reduction appeared to be proportional to the length of decompression, except in the case of animal D-17. The total lipids were consistently increased, and the degree also appeared proportional to the length of decompression. The increase in total lipids was reflected in most instances by increases in total cholesterol and lipid phosphorus.

Sections of the lungs showed evidence of fat embolization only rarely. In most of the animals there was alveolar thickening and congestion. When embolic fat was seen, it was frequently localized in the small arterioles.

TABLE I

Lipid Responses and Post-heparin Clearing Activity Reduction in Rabbits Subjected to Decompression

Animal #	Hours of Decomp.	$\bar{p}$ Heparin Clearing Activity % Reduction	Total lipids mg. %			Total cholesterol mg.%			Lipid phosphorus mg. %		
			Control	After Decomp.	% Change	Control	After Decomp.	% Change	Control	After Decomp.	% Change
D-16	24	39	192	360	+ 87	78	105	+35	4.5	7.5	+66
D-26	24	21	192	327	+ 70	77	77	0	3.4	4.5	+32
D-17	25	83	247	484	+ 96	84	95	+ 13	5.0	7.0	+40
D-21	49	62	304	913	+200	105	262	+140	5.0	13.5	+170
D-5	51	65	227	500	+ 120	30	82	+173	1.5	6.5	+333
D-12	74	78	293	778	+ 166	79	187	+ 137	4.7	12	+155
D-15	96	67	270	848	+214	91	249	+ 174	6.0	16	+167
D-22	96	73	180	800	+344	69	183	+ 165	3.6	11	+205

Animals Succumbing to Decompression Hypoxia

Eleven rabbits subjected to decompression died during the procedure and were excluded from the "clearing" and lipid studies. These animals died one to 12 hours after decompression was begun. The lungs were examined histologically, and regularly revealed marked congestion, effusion and hemorrhage. Many arterioles and capillaries were filled with sudanophilic material; however, the arterioles appeared to be more frequently affected (Figs. 7 and 8). In the larger arterioles and in capillaries cut tangentially, blood cellular elements were noted to be associated with the sudanophilic material (Fig. 8). Birefringent, crystalline material similar to that described in the human necropsy tissue was also demonstrated in the embolic fat of these animals (Fig. 8). In general, the animals which succumbed to decompression hypoxia manifested marked evidence of pulmonary tissue damage and much more frequent occlusion of the blood vessels with fat.

DISCUSSION

Birefringent, crystalline material was observed to be incorporated with embolic fat in the vasculature, especially that of the lungs, of 9 patients in whom a diagnosis of fat embolism was established by histologic examination. Similar lesions were found in rabbits which succumbed to decompression to altitude. A review of the literature revealed no previous report of the association of embolic fat with birefringent crystals. The birefringence and the positive Schultz test indicated that this substance was cholesterol or cholesterol esters.[10] Its presence in tissues following the digitonin reaction and subsequent exposure to an alcohol-ether mixture indicated that the crystals were composed, at least in part, of free cholesterol.[12] The presence of

esterified cholesterol was indicated by the observation that the embolic fat and some of the birefringent material which gave positive reactions to the Schultz test were soluble in an alcohol-ether mixture following digitonin precipitation.[12]

It is necessary for fat to contain from 10 per cent to about 30 per cent cholesterol before it reacts in positive manner to the Schultz test.[10] Since depot fat contains less than one per cent cholesterol,[11] these histochemical observations indicate that depot fat was not responsible for the embolic fat seen in our cases and that other sources are important in fat embolism. The occurrence of fat embolism in a variety of conditions such as trauma without fractures,[20] inhalation anesthesia,[21] decompression to altitude, and caisson disease,[13] casts further doubt on the assumption that depot fat is the primary source of embolic fat.

An obvious source which could account for the amount of cholesterol seen in our study is that present in the circulating blood itself, provided that its aggregation and localization could be explained. A change in the emulsification of the blood lipids as a result of shock or tissue injury has been suggested. According to Pollack, hypoalbuminemia accompanying shock facilitates the precipitation of hydrophobic colloids such as cholesterol.[22] In addition, aggregation of blood cellular elements is known to occur following trauma or stress[23] and even following alimentary lipemia.[24] Davis and Musselman[6] have suggested that the blood lipids may become incorporated into these aggregates and impart the staining characteristics of fat emboli to them.

Other factors which may enhance the aggregation of blood lipids following trauma or shock are an increased accumulation and a deficient utilization of the blood lipids. An increase in blood lipids, including cholesterol, has been reported to follow various forms of stress and trauma.[25-31] Werthessen and Schwenk[32] indicated that increased synthesis of cholesterol occurred after trauma and anoxia of the liver. An increase in blood lipids has also been noted following hemorrhage or the introduction of hemolytic agents in rabbits.[33-36] Starup,[37] investigating this problem, demonstrated an increase in blood lipids by merely subjecting rabbits to prolonged hypoxia in decompression.

A defect in lipid metabolism may also be responsible for their increased accumulation in the blood. Spitzer and Spitzer[38] reported that hemorrhagic lipemia was accompanied by a decrease in heparin-induced lipemia clearing activity. One of us showed that clearing activity following the introduction of heparin was reduced in dogs

during acute hypoxia brought about by rebreathing or by sodium cyanide.[15]

In the animal experiments reported, heparin-induced clearing activity was diminished in rabbits subjected to decompression; in these animals the serum lipids were increased. Both the reduction in clearing activity and the increase in serum lipids appeared to be related to the duration of decompression. The increase in serum lipids and the reduced ability to form clearing factor following the introduction of heparin in animals subjected to decompression suggests that there is an increased mobilization of lipids together with a defect in the metabolism of lipids. This is in accord with the suggestions of Spitzer and Spitzer[38] in their experiments with hemorrhagic lipemia.

The relative lack of fat embolism and tissue damage in the lungs of animals surviving prolonged decompression supports the conclusion that fat embolism is not the result of increased serum lipid *per se*. This observation parallels the clinical experience of Johnson and Svanborg[7] that patients with hyperlipemia were not unusually prone to fat embolism.

It is proposed, therefore, that aggregation of blood lipids results from a deficiency or temporary inactivation of plasma "emulsifiers." In addition, an increase in blood lipids, whether from absorption, traumatized fat depots, increased mobilization, or deficient utilization, would further contribute to the colloidal instability of these lipids.

The extraordinary and predominant localization of these lipid aggregates in pulmonary vessels in both human necropsy tissue and that from experimental animals demands further comment. The lung has been assumed to be the primary organ of localization because it is here that embolic fat encounters the first capillary bed. In our experience with embolization in dogs by injecting olive oil tagged with radioactive iodine (I^{131}) into a peripheral vein, the lung was always the primary site of localization.[39] However, when small amounts of fat were injected into the carotid artery, it traversed the peripheral vascular bed and also localized in the lung.[39] This was apparently true for injection into the renal artery as well. When fat was injected into the portal vein or splenic artery, the primary localization was within the intrinsic vessels of these organs. The pressure differences in the various vascular beds may offer a reasonable explanation of this localization. From available information, it is known that mean capillary pressures are significantly lower in the lung and liver than in the brain, kidney, and peripheral vascular beds.[40] Factors which cannot be overlooked in the localization of embolic fat are the intrinsic nature of the particular endothelium itself, the association

of embolic fat with cellular aggregates, and the possibility that lipids aggregate in areas of vascular damage, much like thrombus formation.

Even though the lung is the primary site of localization, other organs, particularly the brain, are not spared. Swank and Dugger[41] suggested that embolic fat seen in the brain and other tissues originated from emboli released from the lung as the result of vascular atony accompanying shock. In addition, it is known that pulmonary arterial pressure increases following embolization of the lung[42] or in association with hypoxia.[43] This increase in pressure could also be important in pushing emboli into the general circulation to lodge subsequently in other organs. The possibility that embolic fat released from the lung will localize in peripheral vascular beds, while fat injected intra-arterially will traverse these vessels suggests that during localization in the lung, physicochemical alterations in the aggregates enhance their ability to stick and build up in capillary beds subsequently encountered.

On the basis of the above, it is proposed that: (a) fat from traumatized depots can on occasion enter the circulation and produce mechanical embolization of the lung; (b) this process can be of sufficient degree in itself to produce symptoms of fat embolism, but is not the only mechanism involved; (c) the tissue injury, usually associated with shock, can initiate physicochemical alterations resulting in a colloidal instability of plasma lipids; (d) the tissue injury can initiate an increased mobilization and deficient utilization of lipids which is reflected by an increase in plasma lipid; (e) in an unstable colloidal menstruum, the lipids can aggregate and be localized primarily by the pulmonary vasculature; (f) an increase in pulmonary arterial pressure together with vascular atony can cause some of these aggregates to be pushed into the systemic circulation and be localized in other organs; (g) the phenomenon of lipid aggregation and localization occurs to some degree in the majority of traumatic injuries. Under appropriate circumstances, the phenomenon can be of such magnitude or rapidity as to produce a clinically recognizable syndrome with entirely characteristic pathologic features.

Summary

1. Birefringent crystalline material was observed in association with masses of intravascular fat in 9 patients with fat embolism.

2. By histochemical tests, both the crystalline material and the embolic fat were found to contain free and esterified cholesterol in amounts greater than that contained in depot fat.

3. Exposure of animals to decompression, a situation which may

be followed by fat embolism in human subjects, resulted in a defect of lipid metabolism. This was manifested by an increase in serum lipids and an inability to form lipemia clearing factor following the introduction of heparin.

4. In animals surviving decompression, fat embolism was insignificant, while in animals succumbing to decompression, the pathologic pattern was similar to that in the human subjects.

5. In view of these observations, a pathogenesis of fat embolism is suggested.

REFERENCES

1. Peltier, L. F. An appraisal of the problem of fat embolism. *Surg. Gynec. & Obst.*, 1957, **104**, (Internat. Abstracts Surgery), 313–324.
2. Lindsay, S., and Moon, H. D. Bone-marrow embolism following fracture. *J. Bone & Joint Surg.*, 1946, **28**, 377–380.
3. Schenken, J. R., and Coleman, F. C. Bone marrow and fat embolism following fracture of the femur. *Am. J. Surg.*, 1943, **61**, 126–127.
4. Lehman, E. P., and Moore, R. M. Fat embolism; including experimental production without trauma. *Arch. Surg.*, 1927, **14**, 621–662.
5. Whiteley, H. J. The relation between tissue injury and the manifestations of pulmonary fat embolism. *J. Path. & Bact.*, 1954, **67**, 521–530.
6. Davis, H. L., and Musselman, M. M. Blood particle agglomeration and fat embolism. *Internat. Rec. Med.*, 1954, **167**, 439–448.
7. Johnson, S. R., and Svanborg, A. Investigations with regard to the pathogenesis of so-called fat embolism. Serum lipids and tissue esterase activity and the frequency of so-called fat embolism in soft tissue trauma and fractures. *Ann. Surg.*, 1956, **144**, 145–151.
8. Yoffey, J. M., and Baxter, J. S. The formation of birefringent crystals in the suprarenal cortex. *J. Anat.*, 1947, **81**, 335–342.
9. Claesson, L., and Hillarp, N.-A. Critical remarks on the histochemical reactions for ketosteroids. *Acta anat.*, 1947, **3**, 109–114.
10. Palay, S. L. (ed.) Frontiers in Cytology. Yale University Press, New Haven, 1958, pp. 241–243.
11. Kritchevsky, D. Cholesterol. John Wiley and Sons, Inc., New York, 1958, p. 281.
12. Feigin, I. A method for the histochemical differentiation of cholesterol and its esters. *J. Biophys. & Biochem. Cytol.*, 1956, **2**, 213–214.
13. Haymaker, W., and Johnston, A. D. Pathology and decompression sickness. A comparison of the lesions in airmen with those in caisson workers and divers. *Mil. Med.*, 1955, **117**, 285–306.
14. Brown, R. K.; Boyle, E., and Anfinsen, C. B. The enzymatic transformation of lipoproteins. *J. Biol. Chem.*, 1953, **204**, 423–434.
15. LeQuire, V. S.; Worley, L. M., and Gray, M. E. The effects of hypoxia upon the lipemia-clearing response to intravenous heparin. *J. Lab. & Clin. Med.*, 1957, **49**, 869–876.
16. Bloor, W. R. The determination of small amounts of lipid in blood plasma. *J. Biol. Chem.*, 1928, **77**, 53–73.
17. Bloor, W. R. The determination of cholesterol in blood. *J. Biol. Chem.*, 1917, **29**, 437–445.

18. Youngburg, G. E., and Youngburg, M. V. Phosphorus metabolism. I. A system of blood phosphorus analysis. *J. Lab. & Clin. Med.*, 1930–1931, **16**, 158–166.

19. Fiske, C. H., and Subbarow, Y. The colorimetric determination of phosphorus. *J. Biol. Chem.*, 1925, **66**, 375–400.

20. Lehman, E. P., and McNattin, R. F. Fat embolism. II. Incidence at postmortem. *Arch. Surg.*, 1928, **17**, 179–189.

21. Culver, H., and Baker, W. J. Fat embolism following pyelotomy for stone with leukoplakia of the renal pelvis. *Tr. Am. A. Genito-Urin. Surgeons*, 1930, **23**, 151–161.

22. Pollak, O. J. An etiologic concept of atherosclerosis based on study of intimal alterations after shock. *Circulation*, 1952, **5**, 539–550.

23. Knisely, M. H.; Bloch, E. H.; Eliot, T. S., and Warner, L. Sludged blood. *Tr. Am. Therap. Soc.*, 1948, **48**, 95–112.

24. Cullen, C. F., and Swank, R. L. Intravascular aggregation and adhesiveness of the blood elements associated with alimentary lipemia and injections of large molecular substances. Effect on blood-brain barrier. *Circulation*, 1954, **9**, 335–346.

25. Shedlovsky, T., and Scudder, J. A comparison of erythrocyte sedimentation rates and electrophoretic patterns of normal and pathological human blood. *J. Exper. Med.*, 1942, **75**, 119–126.

26. Chanutin, A., and Gjessing, E. C. Electrophoretic analyses of sera of injured dogs. *J. Biol. Chem.*, 1946, **165**, 421–426.

27. Gjessing, E. C.; Ludewig, S., and Chanutin, A. Fractionation, electrophoresis, and chemical studies of proteins in sera of control and injured dogs. *J. Biol. Chem.*, 1947, **170**, 551–569.

28. Milch, L. J.; Redmond, R. F.; Calhoun, W. W., and the Cardiovascular Research Group. Blood lipoproteins in traumatic injury. *J. Lab. & Clin. Med.*, 1954, **43**, 603–614.

29. Milch, L. J.; Redmond, R. F., and Calhoun, W. W. Plasma lipoprotein changes induced by acute local cold injury. *Am. J. M. Sc.*, 1953, **225**, 416–420.

30. Thomas, R. S.; Vaughan, B. E.; Walker, E. L., and Pace, N. Plasma lipoprotein changes following thermal injury in the dog. *Proc. Soc. Exper. Biol. & Med.*, 1954, **85**, 553–558.

31. Seifter, J., and Baeder, D. H. Relation of endocrines and clearing factor inhibitors to hyperlipemia in fasted animals. *Proc. Soc. Exper. Biol. & Med.*, 1956, **93**, 63–67.

32. Werthessen, N. T., and Schwenk, E. Biosynthesis of cholesterol. Factors regulating biosynthesis of cholesterol-like substances in isolated whole livers. *Am. J. Physiol.*, 1952, **171**, 55–61.

33. Chamberlain, E. N., and Corlett, R. L. Cholesterol variations in experimental anaemias. *Brit. J. Exper. Path.*, 1932, **13**, 299–310.

34. Horiuchi, Y. Studies on blood fat. Lipemia in acute anemia. *J. Biol. Chem.*, 1920, **44**, 363–379.

35. Boggs, T. R., and Morris, R. S. Experimental lipemia in rabbits. *J. Exper. Med.*, 1909, **11**, 553–560.

36. Milne, L. S. Über Blutungsanämie. *Deutsches Arch. klin. Med.*, 1913, **109**, 401–409.

37. Starup, U. Einige Untersuchungen über die hämorrhagische Lipämie. *Biochem. Ztschr.*, 1934, **270**, 74–92.

38. Spitzer, J. J., and Spitzer, J. A. Hemorrhagic lipemia: a derangement of fat metabolism. *J. Lab. & Clin. Med.*, 1955, **46**, 461–470.

39. Cobb, C. A., Jr.; LeQuire, V. S.; Gray, M. E., and Hillman, J. W. Therapy of traumatic fat embolism with intravenous fluids and heparin. *Surgical Forum*, 1958, **9**, 751–756.

40. Bard, P. (ed.) Medical Physiology. C. V. Mosby Co., St. Louis, 1956, ed. 10, pp. 41; 206; 222; 790.

41. Swank, R. L., and Dugger, G. S. Fat embolism. A clinical and experimental study of mechanisms involved. *Surg. Gynec. & Obst.*, 1954, **98**, 641–652.

42. Daley, R.; Wade, J. D.; Maraist, F., and Bing, R. J. Pulmonary hypertension in dogs induced by injection of lycopodium spores into pulmonary artery, with special reference to absence of vasomotor reflexes. *Am. J. Physiol.*, 1951, **164**, 380–390.

43. Green, H. D. (ed.) Shock and Circulatory Homeostasis. Transactions of the Fourth Conference. Josiah Macy, Jr. Foundation, New York, 1954, pp. 166–207.

[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Fat embolism in pulmonary vessels in a human patient. Frozen section, stained with Sudan IV and hematoxylin, and examined for birefringence. Arrows indicate masses of sudanophilic material. Blue filter. × 100.

FIG. 2. A pulmonary arteriole containing birefringent crystals. Frozen section, hematoxylin and Sudan IV stains. Blue filter. × 200.

FIGS. 3 and 4. The same arteriole shown in Figure 2, examined under higher magnification. The acicular crystals in the lumen, visible with ordinary light (Fig. 3) exhibit characteristic double refractility when examined with crossed Nicol prisms (Fig. 4). × 430.

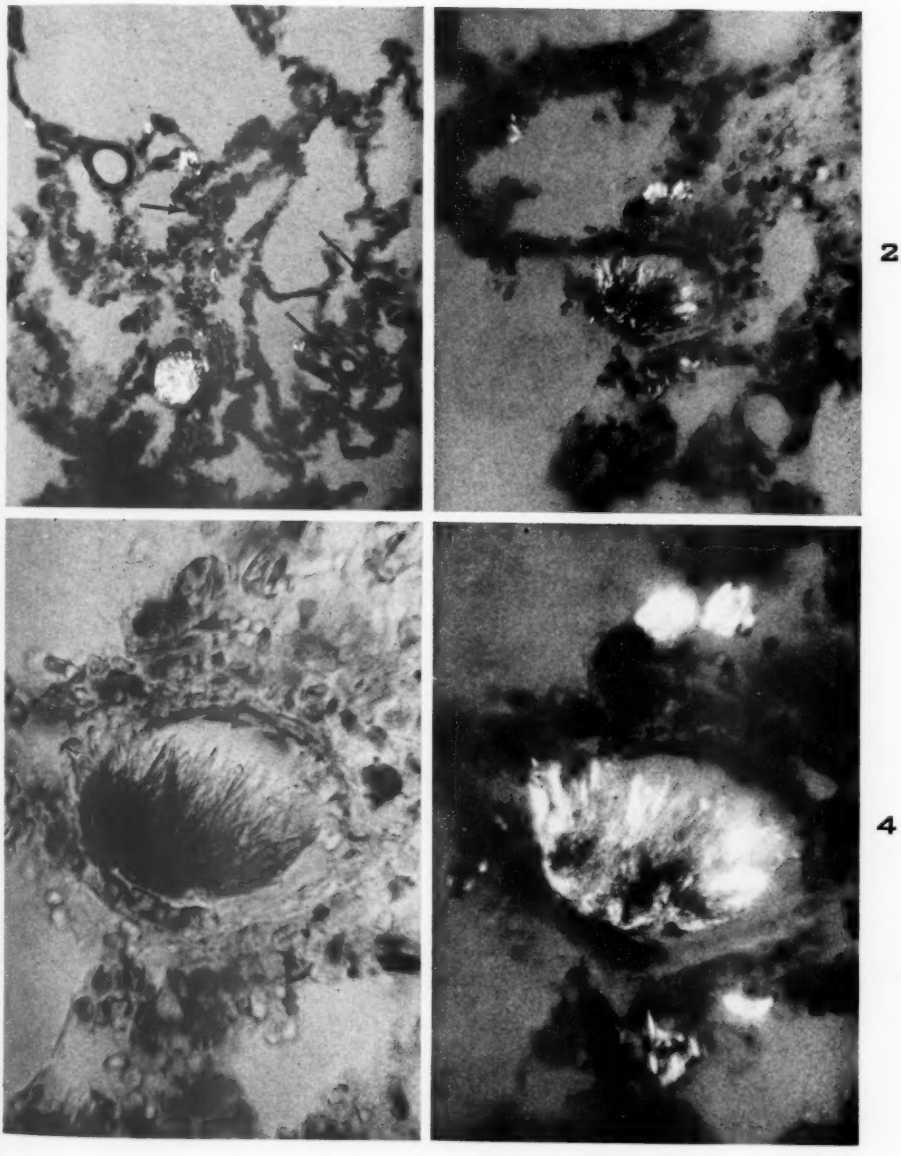
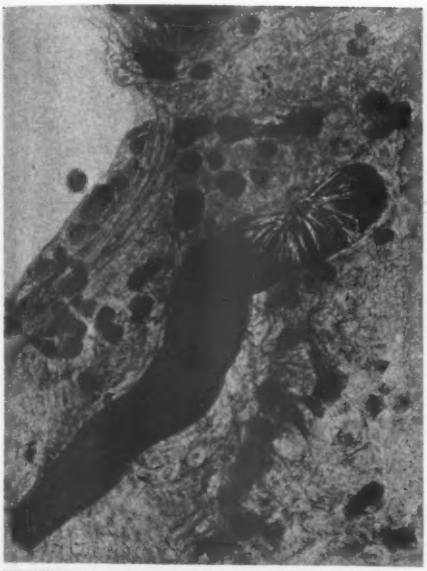


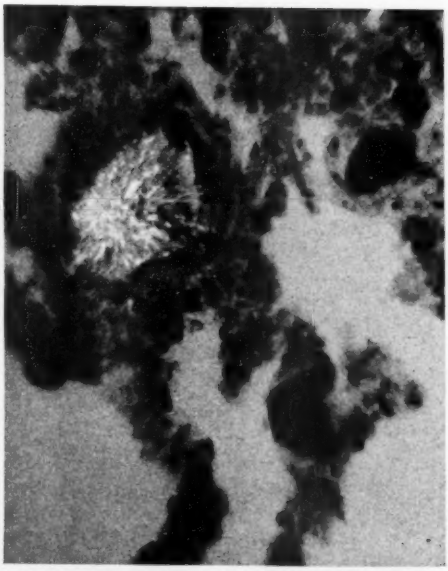
FIG. 5. An artist's drawing of pulmonary fat embolism to demonstrate the acicular character of the crystalline material.

FIG. 6. A photomicrograph of the same area depicted in Figure 5. Note the relationship of the crystalline mass to the embolic fat in the vessel cut tangentially. Frozen section, stained with Nile blue sulfate. × 430.

FIGS. 7 and 8. Demonstrations of the characteristics of fat embolism in the lung of a rabbit dying 1.5 hours after decompression to 350 mm. of Hg. Doubly refractile crystalline material is noted interspersed in the dark-staining neutral fat. Cellular aggregates in association with embolic fat are shown in Figure 8. Hematoxylin and Sudan IV stains. × 430.

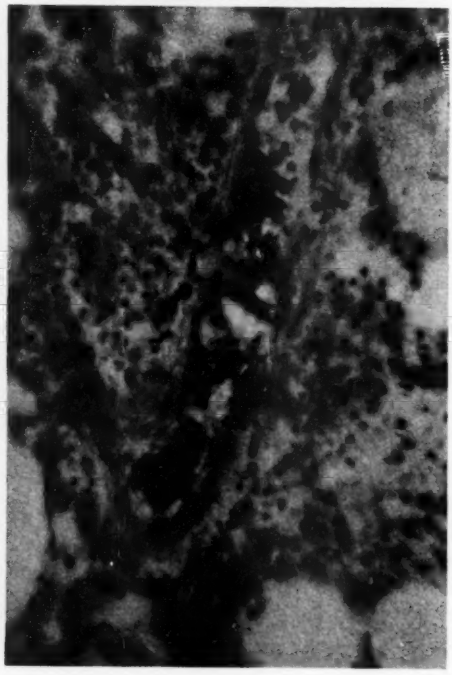


5



7

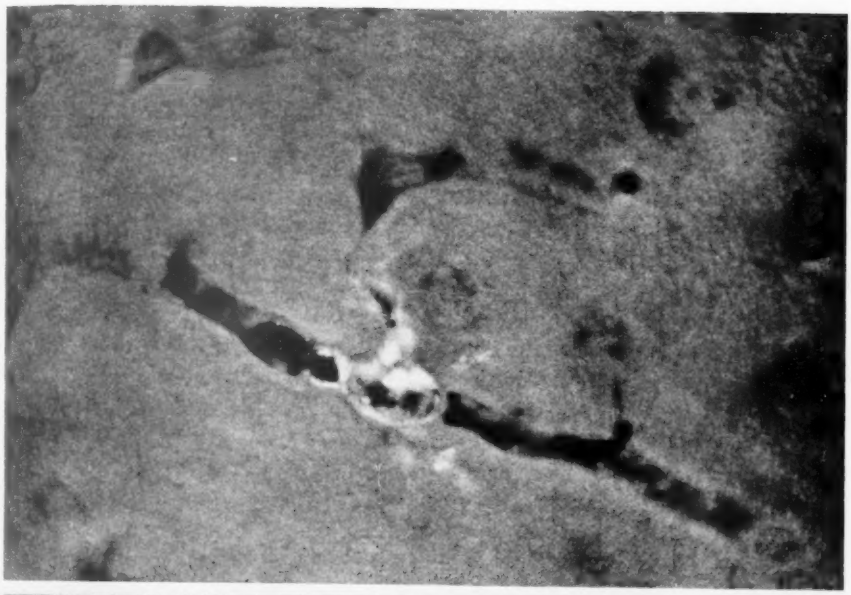
6



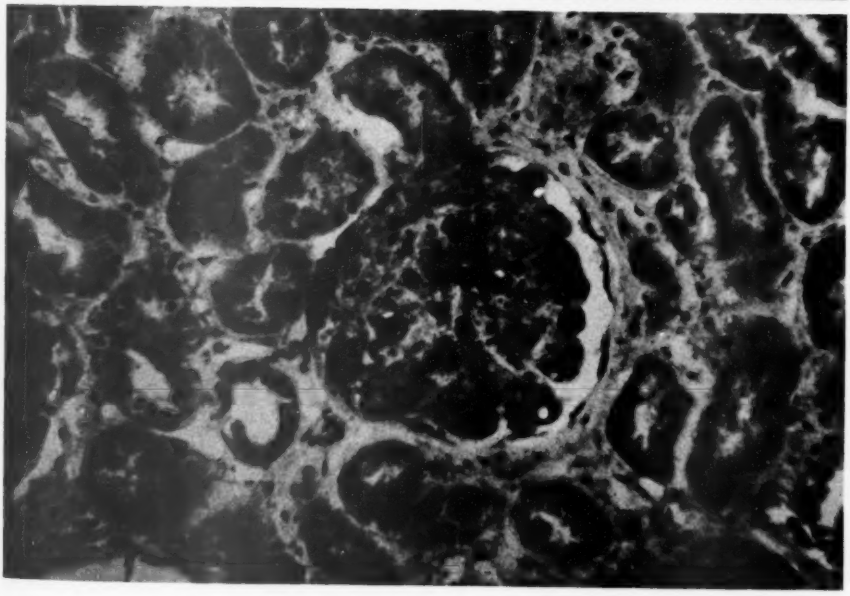
8

FIG. 9. Fat embolism in cerebral vessels of a human subject, to demonstrate mass of birefringent crystals incorporated in dark-stained neutral fat. Note bulbous dilatation of vessel at the site of doubly refractile material. Frozen section, Nile blue sulfate and methylene blue stains. × 430.

FIG. 10. Renal glomerulus from a patient with fat embolism. Dark-staining masses of neutral fat fill many capillaries. There is a small accumulation of doubly refractile material at one o'clock. Hematoxylin and Sudan IV stains. × 200.



9



10

CICATRIX FORMATION IN RAT CEREBRAL CORTEX AS REVEALED BY ELECTRON MISCOSCOPY*

ROBERT L. SCHULTZ, Ph.D.,† and DANIEL C. PEASE, Ph.D.
From the Department of Anatomy, School of Medicine, University of California, and the Veterans Administration Center, Los Angeles, Calif.

The mechanism of cerebral scarring has been well investigated by light microscopy, with the works of Penfield and his associates[1-5] serving as outstanding examples. Most of these studies were done in the period from 1920 to 1930. Since then there have been few published reports dealing with cicatrix formation in the brain. With the introduction of electron microscopy, it seemed desirable to exploit the potentialities of the instrument in an investigation of cerebral wound repair. There were several points which we hoped to elucidate, as, for example, the nature of the glial cells in gliosis, the interrelations between nervous and connective tissue elements, and particularly the barrier between these two.

The electron microscope is a recent innovation, and adequate techniques for employing it in tissue research have been available for only a few years. Because of this there have been no published electron microscopic studies of experimental cerebral lesions previous to this investigation. A general survey of normal cerebral structure, carried out in this laboratory,[6-8] served as a basis for this study.

MATERIAL AND METHODS

Adult rats were used in these experiments. To produce lesions, two small holes were drilled through the skull in a dorsolateral position, one on each side, by means of a dental drill. With caution and practice it was possible to drill these holes without penetrating the dura. A Bard-Parker "stab" blade (size 11) was inserted into the holes as far as it would go. This caused the blade tip to penetrate to a depth of about 1 mm. into the cortex. Recovery intervals of 24, 48, 72 and 96 hours, and 5, 7, 14, 30, 60, 90, 217 and 225 days elapsed after injury before removal of tissue for examination.

The technique of tissue removal and fixation was the same as that previously described for normal cortex.[6] Briefly, this consisted of surgical exposure of the desired region and removal of the dura, followed by fixation with chilled and buffered osmium tetroxide solution *in situ* for 10 minutes. The specimen was undercut, removed, and

* Received for publication, February 19, 1959.
† Present address: Department of Anatomy, College of Medical Evangelists, Loma Linda, Calif.

fixed for 20 minutes more. If the core of the lesion happened to adhere to the meninges as the latter were stripped away from the underlying nervous tissue, this was also preserved as a separate block, and subsequently sectioned. Dehydration was accomplished quickly (within 30 minutes), and the tissue was blocked and sectioned in butyl methacrylate.

Cicatrix Formation

It would be desirable to discuss the changing character of the experimental lesions as a dynamic process; yet it is not practical to characterize in detail each stage chosen for study. In general, the different cell types will be discussed where appropriate in approximately a temporal sequence.

Acute Phase

Microglia. The cortex in the immediate area of the lesion 24 hours postoperatively became a mass of cellular debris. Only microglia persisted as definite cells. All the other cortical cell types were reduced to fragments. Spaces were present and were seen most commonly along vessels. These probably developed by a dissolution of the astrocytic plasma membranes which normally form the perivascular endfeet.

Microglia quickly underwent a remarkable transformation after the lesion was induced. Within 24 hours processes were shortened, and the cells exhibited a rounded appearance as the cytoplasmic volume was increased. The cytoplasm became much less dense than originally so that nuclei were prominent. The nucleoli became very large and conspicuous transiently, and the Golgi membranes were numerous. As development progressed, however, the nucleoli and Golgi apparatus became less conspicuous. In about one week these changes culminated in the formation of typical "gitter" cells with much pale cytoplasm and no definite processes.

It seems probable that macrophages of hematogenous origin entered the lesion. However, it was not possible to determine by structure alone whether any one gitter cell was of hematogenous or microglial origin.

The cytoplasm of gitter cells became packed with ingested material —so much so, that the nucleus was often pushed aside to the cell periphery. The structure of the phagocytosed particles varied widely, some being suggestive of myelin, and others of a more obscure lipoidal or other nature (Figs. 1 and 2). The inclusions in Figure 1 are particularly interesting since they seem to have originally contained 3 separate components. They are not specific for degenerating nervous

tissue, however, for Dmochowski[9] has shown inclusions which appear identical in the thymus gland of leukemic mice. Commonly, an area close to the nucleus, the juxtanuclear zone, was devoid of inclusions. Golgi membranes were prominent in this region. Mitochondria of the usual type could be seen throughout the cytoplasm. The cytoplasmic margin was often irregular with processes (pseudopods) extending for short distances into the surrounding debris. This had an appearance typical of a mobile, ameboid cell.

Gitter cells dominated the lesion for approximately the first week and then began to decrease in number slowly until by 90 days postoperatively only an occasional phagocyte was to be seen.

Neutrophils. Neutrophils were abundant throughout the lesion at 24 hours, but diminished afterward.[10] Often these cells were seen packed into the cellular debris. The presence of this cell type in numbers is usually considered indicative of the acute phase of a pathologic process, and thus it was here, also.

Erythrocytes. The trauma of producing the lesion caused a certain amount of bleeding. Thus red cells were to be seen frequently. It was surprising how long these persisted in the lesion. In this investigation they were observed 60 days postoperatively, still appearing essentially normal. Penfield and Buckley[11] reported them lasting 79 days without disintegration.

Vasculature. Shortly after the lesion was established, the walls of vessels were greatly thickened and extensively damaged. Of the structures normally present, the only features easily recognized were basement membranes; cellular detail was destroyed. Thus it was that numerous and extensive gaps existed in the endothelium. In the lumens, clumps of platelets were often seen.

Subacute and Chronic Phases

Plasmocytes. The plasma cell as seen by electron microscopy has been described by Braunsteiner, Fellinger and Pakesch[12] and is characterized by abundant endoplasmic reticulum which practically fills the cytoplasm. Mitochondria, scattered between cisternas, and Golgi membranes, while definitely present, are inconspicuous. The cartwheel nucleus seen by light microscopy is represented by aggregations of chromatin in the electron micrographs but may not always be obvious. By one month postoperatively, numerous plasma cells were present in the lesion. They tended to form groups, i.e., a number were usually found together in any one locus. Often they were observed adjacent to a vessel as a so-called perivascular cuff.

Remarkable differences in the development of the endoplasmic

reticulum could be seen in these plasma cells (Figs. 3 and 4). The variables were the width of the cisternas, the degree of their packing together, and the orientation of the profiles (ranging from parallel to completely random distribution). All possible combinations were encountered. The substance filling the cisternas of the endoplasmic reticulum as swelling occurred came to have a substantial density. Thus it was not just imbibed water, but probably represented precipitated protein (Fig. 4). As the cisternas became enlarged, the remaining cytoplasm became reduced to thin sheets. Mitochondria in such thin cytoplasmic compartments produced conspicuous bulges. Eventually such cells seemed to disintegrate and formed small spherical bodies containing the substance previously within the cisternas (Fig. 3, arrows). These bodies were enclosed by a membrane with granules attached to their outer surfaces. Apparently the membranes and granules represented the residua of the endoplasmic reticulum and its associated ribonucleic acid (RNA) granules.

The most likely and interesting implication of this activity was that antibodies were being formed and set free. Kolouch[13] identified the plasma cell with allergic phenomena by producing hypersensitivity in rabbits and observing the resulting development of plasmocytes in the bone marrow.

Lymphocytes and Eosinophils. Lymphocytes were conspicuously absent and were observed with certainty only occasionally. This fact is of some interest, particularly when one considers that plasma cells, which were numerous in the lesion, are generally thought to arise from lymphocytes.

Eosinophils were more frequently encountered. Unfortunately, it was not possible to come to any conclusions regarding the significance of this cell type.

Glial Cells. A definite identification of glial cells in old lesions was difficult at best and was often impossible (Figs. 5 and 7). At one extreme, one could find large cells with light, watery cytoplasm and pale nuclei, which resembled the astrocytes of normal cortex as seen by electron microscopy.[6,14] Smaller cells with features of oligodendroglia, characterized by denser cytoplasm and darker nuclei, constituted the other extreme. A spectrum of intermediate cell types was manifest.

The existence of intermediate glial types is not surprising, for both astrocytes and oligodendrocytes are thought to differentiate from the same stem cell, the spongioblast. Thus, as the result of the stimulus of injury, normal macroglia probably revert to a more primitive (embryonic) state. Similar intermediate cell forms have been observed

in the corpus callosum of young rats 13 and 18 days old, and reported elsewhere.[6] Penfield[1] has indicated that macroglia are not completely differentiated until long after birth.

As the lesion aged, definitive astrocytes became arranged so as to form a nearly complete barrier between the cortex on one side and leptomeningeal cells on the other. The latter proliferated into the center of the lesion, forming a core (Figs. 11 and 12). A basement membrane was present between the astrocytic barrier and the core area. This must be formed *de novo* and was unusual in having odd, finger-like projections which indented the adjacent astrocytic cytoplasm (Fig. 12). The processes forming the barrier were very closely fitted together even though the cytoplasmic borders were irregular and occasionally infolded.

The structure of the reconstituted astrocytic "wall" fundamentally resembled that of the subpial astrocytic "membrane" encountered in normal cortex. The general relationships were similar, i.e., cortex on one side and leptomeninges on the other, separated by a basement membrane. However, as indicated in Figures 11 and 12 (217 and 225 days postoperatively), the astrocytic barrier of the lesion was considerably wider than the normal subpial layer. Also no particularly long mitochondria as observed in the normal tissue were noted.[6,14]

Lipogenesis. One of the most interesting features of the older lesions was the presence of large quantities of layered, myelin-like material in the reactive area as early as 30 days postoperatively. Some of this was clearly situated about nerve fibers (Figs. 5, 8 and 9); the remainder, while of identical layered pattern, was found to be associated with glial cells or lay in apparently isolated manner (Figs. 6 and 7). In spite of its erratic and unusual distribution, it seemed reasonable to identify this substance as myelin, for irrespective of its location, it exhibited essentially the same ultrastructure. The fact that the spacing between layers might vary somewhat seemed not to represent a serious objection. In a comprehensive study of myelin, Fernández-Morán and Finean[15] reported that myelin of the central nervous system tended to show irregular spacing. One would expect to find even wider variation under pathologic conditions.

It is important to note that the number of myelinated nerve fibers (Fig. 9) in any given field near the lesion was greatly increased over that seen in the normal cerebral cortex. Thus the origin of these fibers was a matter of some concern. The most likely explanation was that existing cortical neurons became myelinated in response to the nonspecific injury. They could not, of course, represent peripheral nerves which had grown into the lesion, for they were not wrapped by Schwann

cell cytoplasm.[16] In fact, Schwann cells were not observed in the lesion at all. In the unusual pathologic condition *état marbré*,[17] there is also an increase in myelinated nerve fibers apparently arising *de novo* in the cortex. This may be of such a degree that patches of white matter are detected grossly in the cortical gray substance.

Myelin formations can be seen along the cytoplasmic margins of glial cells in Figures 7 and 10. The two cells shown in Figure 7 are intermediate in structure, showing characteristics of both oligodendroglia and astrocytes. The lipoidal material is seen here only along one side of the cells. However, Figure 10 shows that it may completely encircle a cell. This structure, similar in appearance to a normal oligodendrocyte, happens to lie in an artifactitious space close to the core. As in Figure 6, other peculiar formations may be noted; these are not definitely associated with nerve or glia. It is not possible to comment further about their nature.

Meningeal Reactions. Leptomeningeal cells proliferated and migrated into the lesion. By 30 days postoperatively, strands of this cell type were visibly in process of forming a definite core. At this time the penetrating trabeculas were rather loosely organized without definite limits to their extent as in the case of the astrocytic barrier which was established later.

When the cells in the core of the lesion (Figs. 13 to 15) were compared with those of the normal pia-arachnoid,[8] a striking morphologic similarity was evident. The cytoplasm of the cells in the lesion varied from a pale appearance to a moderate density. Normal pia-arachnoid elements more closely resembled the lighter cells of the lesion. Cytoplasmic organelles were identical in both normal and abnormal elements. Cytoplasmic processes were occasionally extremely long (Fig. 13). Often the distribution of the processes was irregular and they could not be traced readily. The manner in which the cytoplasmic borders of adjacent cells were fitted together was of some interest. This is shown in a longitudinal section of a trabecula in Figure 13 and in transverse section in Figure 15. The arrangement is less well oriented in Figure 14. Interdigitation and infolding of cytoplasmic extensions in a curious fashion, much like the pieces of a jig-saw puzzle, are best shown in the latter two figures.

Some spaces were visible between the cells comprising the core of the lesion. Often strands of collagen were seen here. It is to be noted that this is a characteristic of normal pia-arachnoid also. Typical axial periodicity of 640 Å, the established pattern of mature collagen,[18] was manifest, and occasionally, under favorable circumstances, subperiods could be discerned. Sufficient collagen was present between

cells so that the core was often pulled out when the meninges were stripped from the cortex at the time of fixation. At 225 days postoperatively the core consisted of a compact mass of leptomeningeal cells and collagen, limited by an astrocytic barrier and basement membrane. The latter features have already been described. The dura did not seem to take an active role in cicatrix formation under the conditions of this investigation, concerned, as it was, only with small lesions.

Neurons. From the evidence obtained, little can be said about the degeneration and regeneration of neurons. A major difficulty was to determine what represented the result of poor fixation and what was actual alteration. Without doubt those neurons in the immediate region of the lesion became necrotic and were removed by phagocytes. Other neurons more remote from the wound probably recovered, for after 225 days the cortex adjacent to the new astrocytic barrier appeared indistinguishable from normal cortex.

Discussion

The existence of myelin on the surfaces of glial cells, and the appearance of numbers of small myelinated nerve fibers following the induction of cortical lesions were observations having important implications which merit primary consideration. The former feature is one which has not previously been reported, although there are suggestions of the latter phenomenon reported.[17]

Myelin is found about cells identifiable as both astrocytes and oligodendrocytes. This is in agreement with the hypothesis of Luse[19] which proposes that both glial types may be responsible for the formation and maintenance of myelin. As yet, one cannot be certain that the myelin is in reality a product of the cell upon which it is found. However, we are able to affirm that a strong, relatively nonspecific stimulus for the production of myelin certainly must exist.

Gliosis as classically described by light microscopists was not observed. In spite of the fact that the glial barrier surrounding the core of the lesion contained astrocytic processes that were far thicker than usual, the cytoplasm of these cells was essentially comparable to that of normal astrocytes. There are several possible explanations for the absence of gliosis. The most likely, perhaps, is that the lesions described in this report were too small to stimulate abnormal glial fibrogenesis. Obviously, future investigation must explore the events which follow the induction of massive lesions.

Several investigators have noted the formation of connective tissue cores in cortical lesions.[2-5,11,20] Also Clemente and Windle[21] and Scott

and Clemente[22] reported a similar proliferation of pia across the cut ends of the spinal cord. This formed a pia-glial membrane, which was essentially equivalent to the core and astrocytic barrier encountered in this investigation.

It is noteworthy that while no perivascular spaces are present in normal tissue,[7,23,24] they quickly developed after the induction of lesions, as this study has shown. They were formed by a dissolution of the astrocytic end-feet, doubtlessly corresponding to the *"locus minoris resistentiae"* of Kuhlenbeck.[25] In older lesions these spaces became a favored site for plasma cell aggregations.

There is confusion in the published reports concerning the origin of phagocytes in cerebral lesions. Lymphocytes, oligodendroglia, monocytes, vessel adventitial cells, leptomeningeal cells, and microglia have all been suggested as possible precursors. We hoped at the beginning of this investigation to obtain some positive information concerning this matter, but this has not proved possible. The few clues we have seem to indicate that macrophages can arise both from the microglia and from a hematogenous source.

Summary

Small, sterile puncture wounds were made in rat cerebral cortex and the animals sacrificed at intervals from 24 hours to 225 days afterward. An electron microscopic investigation served to compare the pathologic features with normal tissue.

A rapid, acute infiltration of neutrophils occurred within 24 hours and decreased after several days. During this time gross disintegration of cortical tissue was evident. True perivascular spaces appeared; these were not present in normal tissue. Most likely these developed as the result of disintegration of perivascular astrocytic end-feet.

Hematogenous macrophages (monocytes) were noted in and around vessels with distorted, swollen walls. At 48 hours the first activated microglial cells were visible. Both microglia and hematogenous macrophages were thought to contribute to the formation of the fully developed phagocytes of the central nervous system, the "gitter" cells. These increased in number greatly so that they dominated the lesion in one week. Thereafter, their numbers decreased slowly.

A subacute and chronic stage ensued, and was plainly in evidence from 30 days on. Plasma cells of variable structure were very numerous during this period. Vesicles were often noted in close proximity to swollen plasma cells. These seemed to represent surviving remnants of endoplasmic reticulum following plasma cell lysis. This was evidenced by the existence of granule-containing limiting membranes

enclosing the droplets. Swelling and lysis of plasma cells were thought to represent possible mechanisms for the release of antibodies. Concentrations of plasma cells were encountered most often near vessels, forming perivascular cuffs. Eosinophils were occasionally noted, but lymphocytes were conspicuously absent.

Glial cells, in reacting to the lesion, were so altered in structure that often a definite designation was impossible. Thus, while some cells appeared to be astrocytes and others oligodendrocytes, some were intermediate in form and had features of both types. Some cells contained quantities of fine fibrils in their cytoplasm.

Myelin in excess of that encountered normally in the rat cerebral cortex was observed. This not only appeared around nerve fibers but about glial cells and in isolated deposits as well. It was thought that the induction of the lesion constituted a powerful stimulus to myelinogenesis.

As phagocytic activity removed the myelinated structures, and other extraneous cellular material, leptomeningeal cells invaded the lesion, forming a core containing some collagen. Proliferation of the pia-arachnoid could be seen as early as 30 days after injury. Astrocytes formed a thick barrier between the core and the surrounding cortex. The astrocytic cytoplasm was separated from the cells of the core by a basement membrane. Finally a collagenous scar of leptomeningeal derivation marked the site of the lesion.

REFERENCES

1. Penfield, W. Neuroglia and Microglia. The Interstitial Tissue of the Nervous System. In: Special Cytology. The Form and Functions of the Cell in Health and Disease. Cowdry, E. V. (ed.). Paul B. Hoeber, Inc., New York, 1932, ed. 2, Vol. 3, pp. 1445–1482.
2. Penfield, W. G. Meningocerebral adhesions. A histological study of the results of cerebral incision and cranioplasty. *Surg. Gynec. & Obst.*, 1924, **39**, 803–810.
3. Del Rio, H. P., and Penfield, W. Cerebral cicatrix: The reaction of neuroglia and microglia to brain wounds. *Bull. Johns Hopkins Hosp.*, 1927, **41**, 278–303.
4. Penfield, W. The mechanism of cicatricial contraction in the brain. *Brain*, 1927, **50**, 499–517.
5. Foerster, O., and Penfield, W. The structural basis of traumatic epilepsy and results of radical operation. *Brain*, 1930, **53**, 99–119.
6. Schultz, R. L.; Maynard, E. A., and Pease, D. C. Electron microscopy of neurons and neuroglia of cerebral cortex and corpus callosum. *Am. J. Anat.*, 1957, **100**, 369–407.
7. Maynard, E. A.; Schultz, R. L., and Pease, D. C. Electron microscopy of the vascular bed of rat cerebral cortex. *Am. J. Anat.*, 1957, **100**, 409–433.
8. Pease, D. C., and Schultz, R. L. Electron microscopy of rat cranial meninges. *Am. J. Anat.*, 1958, **102**, 301–321.

9. Dmochowski, L. The electron microscope in a cancer hospital. *Scientific Instrument News* (Radio Corporation of America), 1957, **2**, 1–6.

10. Ramon y Cajal, S. Degeneration and Regeneration of the Nervous System. Oxford University Press, London, 1958, Vol. 2.

11. Penfield, W., and Buckley, R. C. Punctures of the brain. The factors concerned in gliosis and cicatricial contraction. *Arch. Neurol. & Psychiat.*, 1928, **20**, 1–13.

12. Braunsteiner, H.; Fellinger, K., and Pakesch, F. Demonstration of a cytoplasmic structure in plasma cells. *Blood*, 1953, **8**, 916–922.

13. Kolouch, F., Jr. Origin of bone marrow plasma cell associated with allergic and immune states in the rabbit. *Proc. Soc. Exper. Biol. & Med.*, 1938, **39**, 147–148.

14. Farquhar, M. G., and Hartmann, J. F. Neuroglial structure and relationships as revealed by electron microscopy. *J. Neuropath. & Exper. Neurol.*, 1957, **16**, 18–39.

15. Fernández-Morán, H., and Finean, J. B. Electron microscope and low-angle x-ray diffraction studies of the nerve myelin sheath. *J. Biophys. & Biochem. Cytol.*, 1957, **3**, 725–748.

16. Geren, B. B. The formation from the Schwann cell surface of myelin in the peripheral nerves of chick embryos. *Exper. Cell Res.*, 1954, **7**, 558–562.

17. Norman, R. M.; Urich, H., and McMenemy, W. H. Vascular mechanisms of birth injury. *Brain*, 1957, **80**, 49–58.

18. Gross, J., and Schmitt, F. O. The structure of human skin collagen as studied with the electron microscope. *J. Exper. Med.*, 1948, **88**, 555–568.

19. Luse, S. A. Formation of myelin in the central nervous system of mice and rats, as studied with the electron microscope. *J. Biophys. & Biochem. Cytol.*, 1956, **2**, 777–784.

20. Wilson, R. B. Brain repair. *Arch. Neurol. & Psychiat.*, 1926, **15**, 75–84.

21. Clemente, C. D., and Windle, W. F. Regeneration of severed nerve fibers in the spinal cord of the adult cat. *J. Comp. Neurol.*, 1954, **101**, 691–731.

22. Scott, D., Jr., and Clemente, C. D. Regeneration of spinal cord fibers in the cat. *J. Comp. Neurol.*, 1955, **102**, 633–669.

23. Patek, P. R. The perivascular spaces of the mammalian brain. *Anat. Rec.*, 1944, **88**, 1–24.

24. Woollam, D. H. M., and Millen, J. W. Perivascular spaces of the mammalian central nervous system. *Biol. Rev.*, 1954, **29**, 251–283.

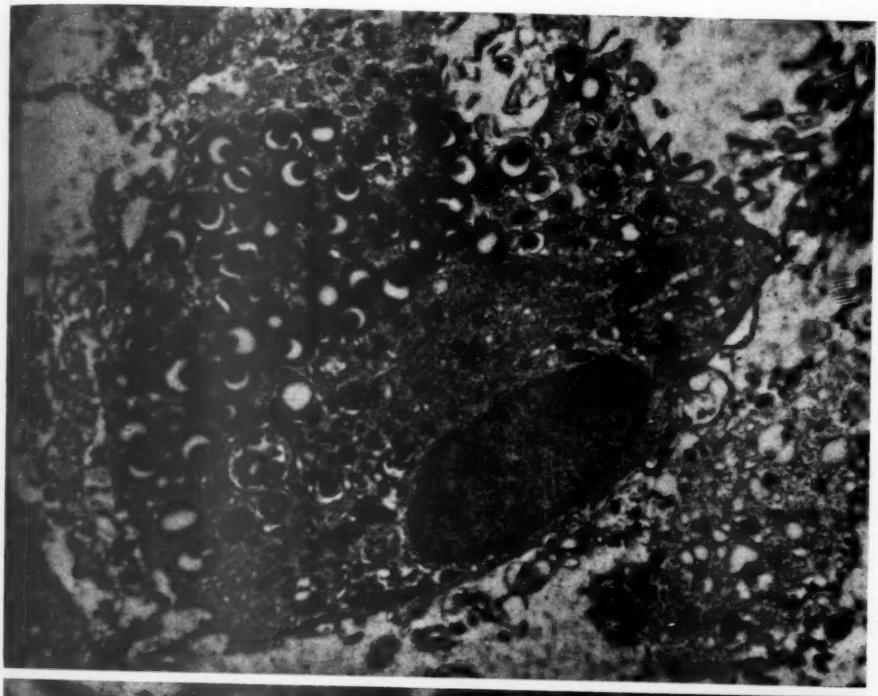
25. Kuhlenbeck, H. Virchow-Robin spaces of His-Held and their relation to the *membrana limitans perivascularis*. (Abstract) *Anat. Rec.*, 1951, **109**, 375.

[*Illustrations follow*]

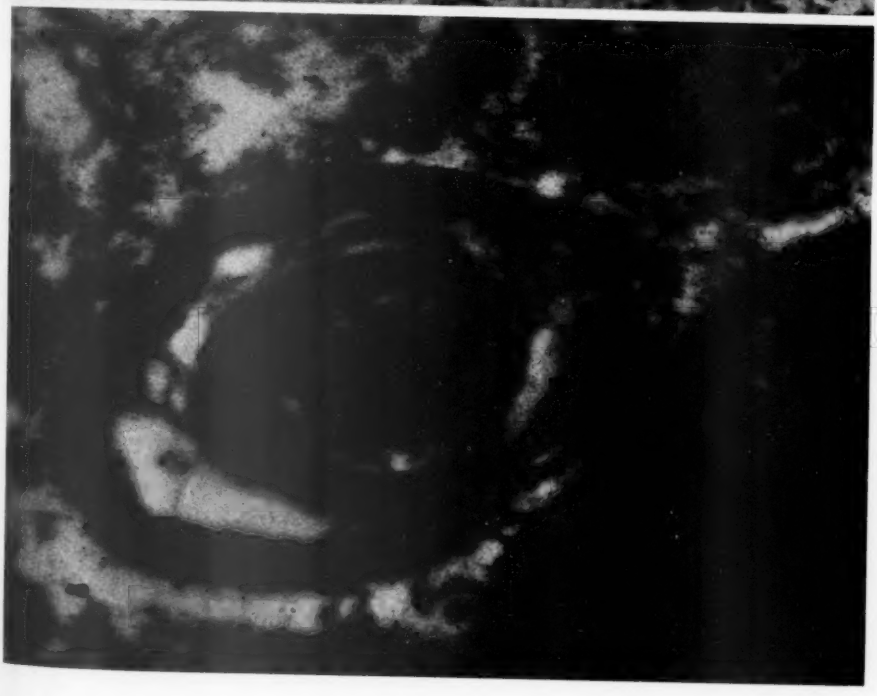
LEGENDS FOR FIGURES

FIG. 1. A typical "gitter" cell. Ingested material fills a major portion of the cytoplasm peripherally. A zone close to the nucleus is devoid of phagocytosed material and contains only a concentration of the usual cytoplasmic organelles. Some of the ingested particles seem to have contained separate components (probably lipid) as evidenced by density differences. The cytoplasmic border is irregular and there are suggestions of pseudopods. 5 days after operation. × 10,500.

FIG. 2. A portion of a macrophage at higher magnification. The whorl-like layered structure at the center is uncommon. The spacing of the layers corresponds to that of myelin. Thus, this formation and the structure immediately to the right of it are possibly derived from myelin. The remainder of the inclusion to the lower right (the dense, granular portion) resembles other frequently observed inclusions. Very small, dense granules are also scattered throughout the cytoplasm. 5 days after operation. × 90,000.



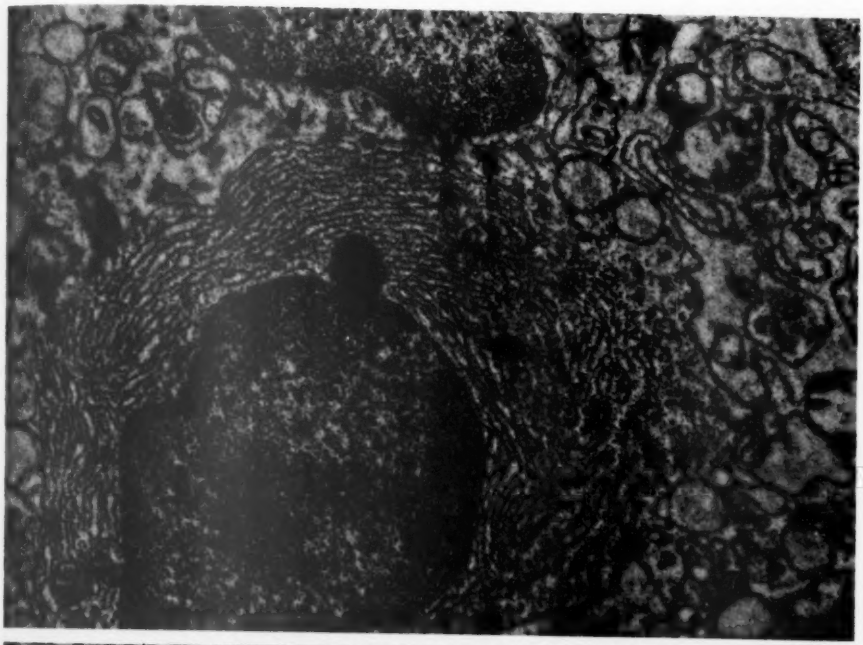
1



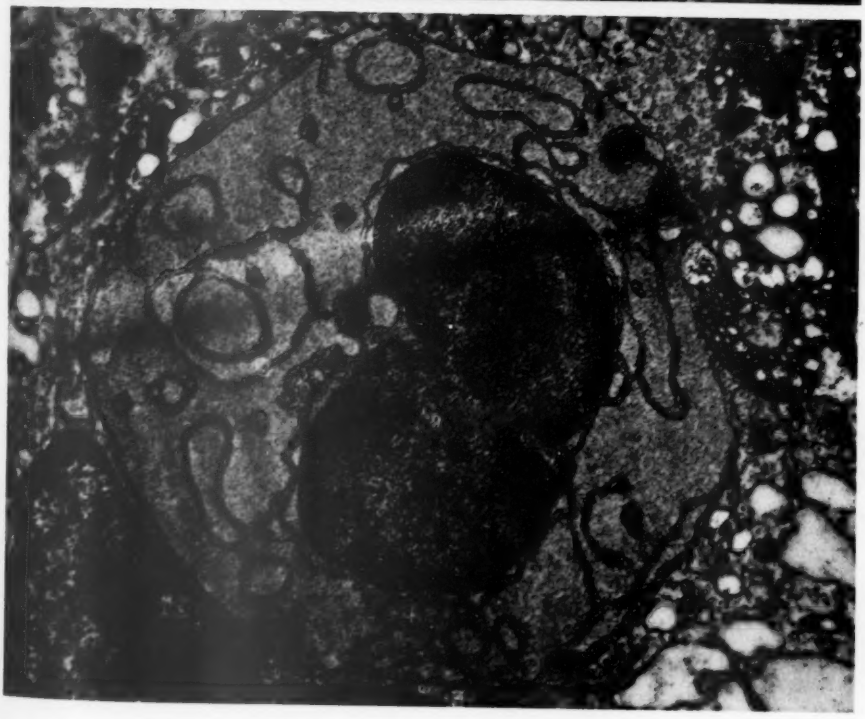
2

FIG. 3. Plasma cells. The plasma cell in the center illustrates the usual form with moderately developed endoplasmic reticulum. The portion of a nucleus at the top margin belongs to a cell which appears to be disintegrating into small vesicles covered by RNA granules (arrows). It is thought that the plasma membrane of the latter cell had disintegrated before fixation. 30 days after operation. × 10,000.

FIG. 4. A plasma cell. The cisternas of the endoplasmic reticulum are enormously swollen. The mitochondria (arrows) are "squeezed" between cisternas so that they form small bulges. The material present in the cisternas is moderately dense and thus does not simply represent imbibed water. 30 days after operation. × 10,000.



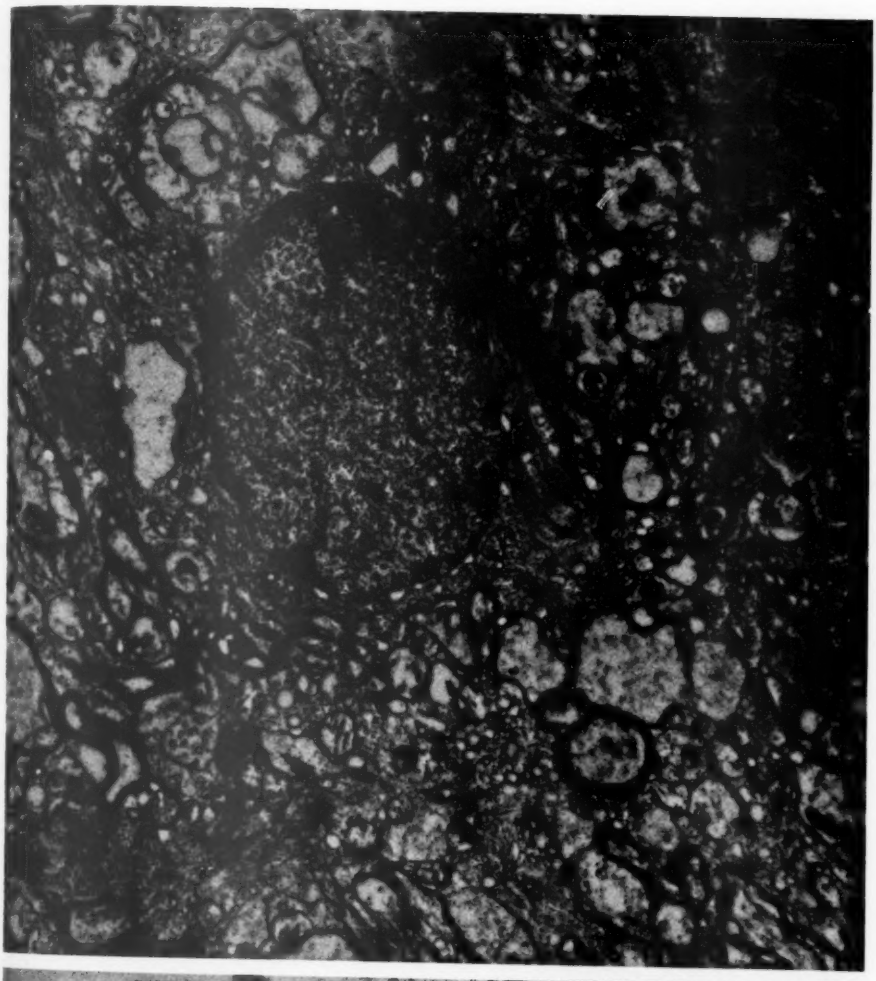
3



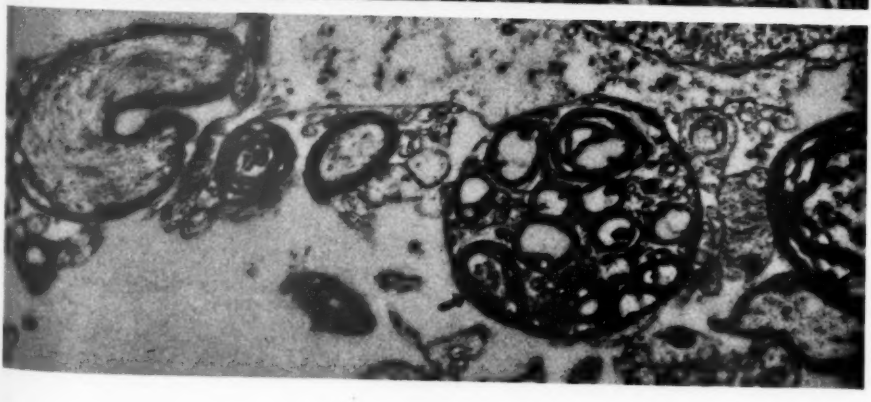
4

FIG. 5. A cell similar to a normal oligodendrocyte in size and density but containing a remarkable number of fibrils (arrows) in its cytoplasm. Similar masses of fibrils have been seen in cells of the developing corpus callosum in young rats. Myelinated formations are closely associated with its borders. Many, if not all, of these structures represent small nerve fibers. Normal rat cortex is notably deficient in myelinated fibers, so that these are thought to represent myelination arising *de novo* and resulting from the stimulus of the lesion. 30 days after operation. × 10,500.

FIG. 6. A curious myelinated structure is indicated by the arrow. This may represent an aberrant formation of axons; however, its true significance remains unknown. The obliquely sectioned nerve fiber at the upper left shows typical neural filaments in its axoplasm. 30 days after operation. × 14,000.

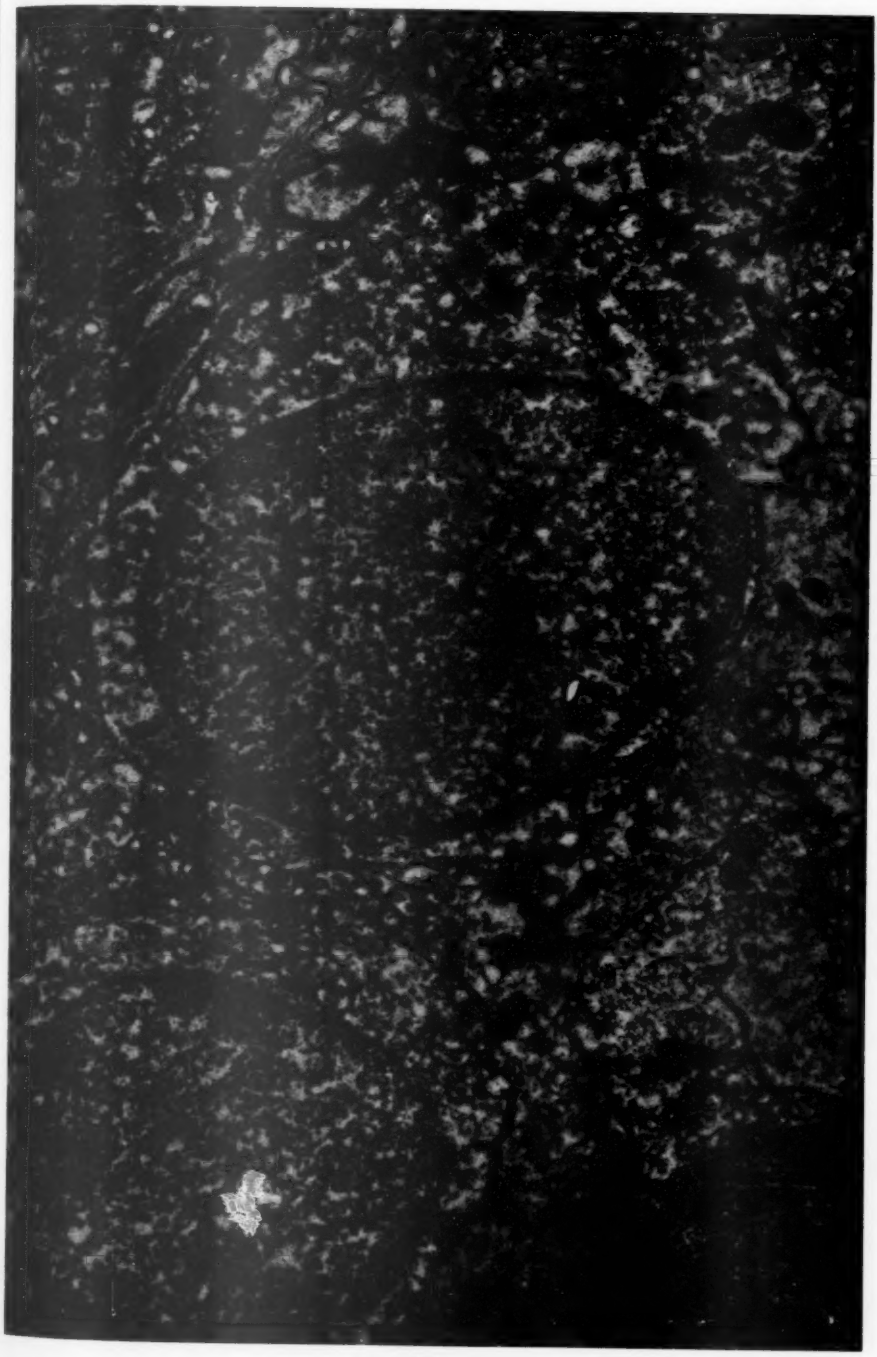


5



6

FIG. 7. Lipid formations are clearly evident along the right cytoplasmic margins of these two cells. This substance, if not myelin, is morphologically similar to it. In the lesion, astrocytes and oligodendroglia often undergo reactive changes, becoming intermediate in structure to the point that a definite designation is impossible. Such is the case here. Glial fibrils are visible in the surrounding processes. 30 days after operation. × 15,500.

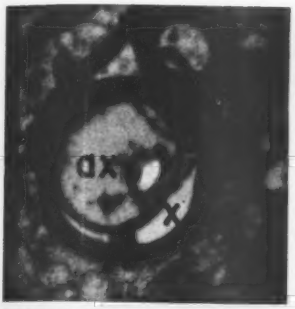


7

FIG. 8. One myelinated axon is shown in transverse section, and parts of several others are less well oriented. Myelinated fibers such as these are much more numerous in the lesion than in normal rat cortex. The axon (ax) of the fiber in the center appears to be shrunken, producing a "space" (x) between it and the myelin sheath. This space is not necessarily an artifact, but may represent glial cytoplasm. 30 days after operation. × 17,700.

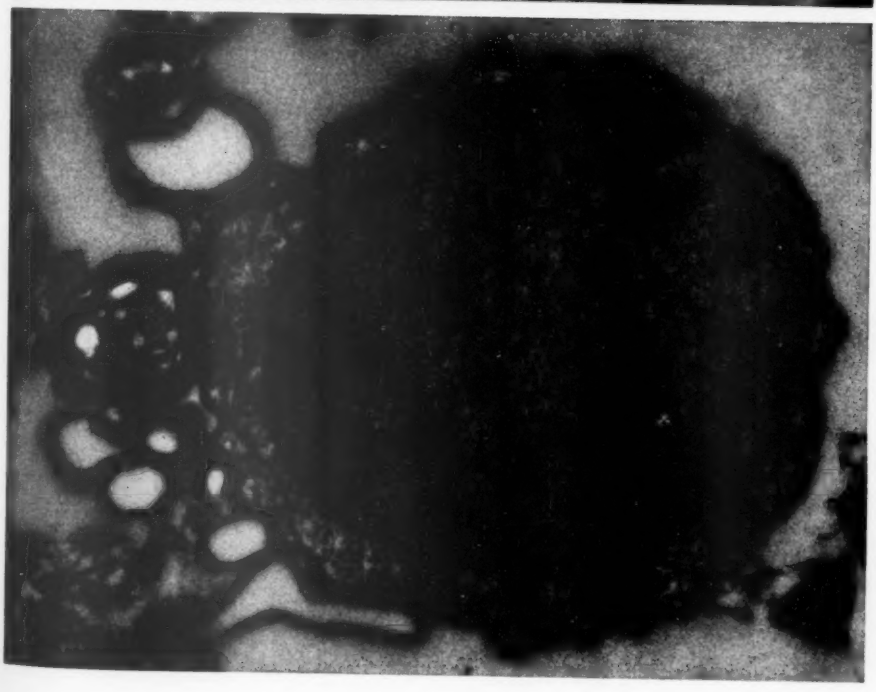
FIG. 9. A portion of the transversely sectioned fiber in Figure 8 at higher magnification. The spacing of the layers measures 100 Å. A layer of cytoplasm (arrows) at least partially encloses the axon and sheath. 30 days after operation. × 106,000.

FIG. 10. Myelin completely surrounds this oligodendroglia-like cell. The space in which it is situated is an artifact due to mechanical distortion in removing the tissue from the animal. The fact that the myelin remains adherent to the cell and completely surrounds it would seem to furnish circumstantial evidence that the cell is the source of the myelin. 225 days after operation. × 17,000.



8

9

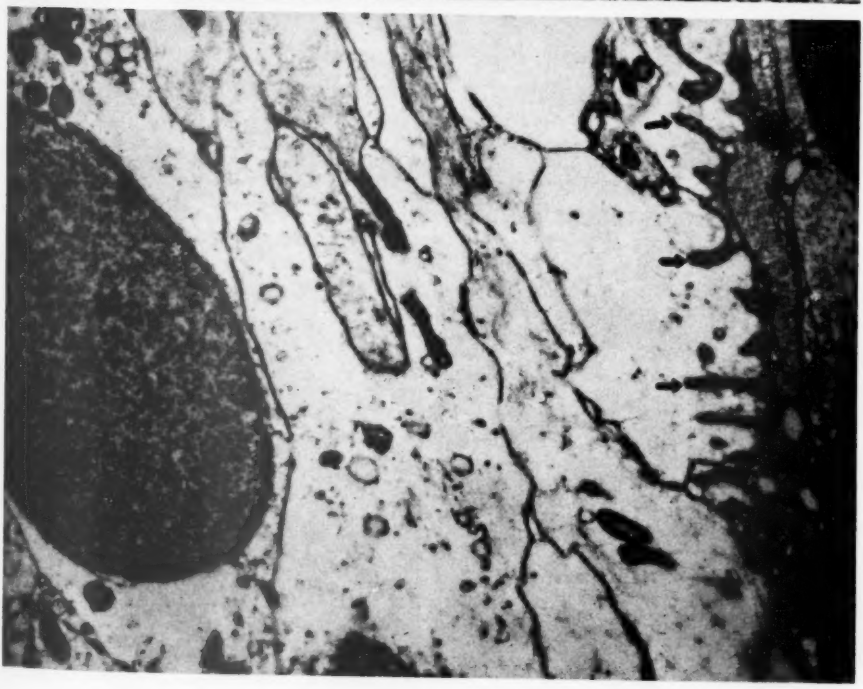


10

FIG. 11. Astrocytic "walling-off" of the core area. A thin strip of cortex (c) is present along the left border of the photograph. Immediately to the right are a number of watery appearing astrocytic processes (ap) forming a barrier between the cortex and the core. This astrocytic layer is much thicker than astrocytic "membranes" (perivascular and subpial) seen in normal cortex. At the astrocytic surface there is a basement membrane too delicate to be visible at this low magnification. Outside of the basement membrane there is often a considerable quantity of precipitated material (pp). Leptomeningeal cells of the core occupy the greater portion of the photograph. The core is well vascularized as evidenced by the vessel in the lower right portion. 217 days after operation. × 7,000.

FIG. 12. The hypertrophied astrocytic barrier of the cortex extends to the left; the core area is on the extreme right. A blood vessel of the core is sectioned longitudinally as it abuts the glial "wall," and the edges of two red cells are visible. A basement membrane between the endothelium of the vessel wall and the astrocytic barrier sends odd finger-like projections into the astrocytic cytoplasm (arrows). A number of glial processes fit together to form the barrier. In some of the processes a few fine fibrils are visible. An astrocytic nucleus is prominently displayed near the left border of the photograph. 225 days after operation. × 11,500.

11



12

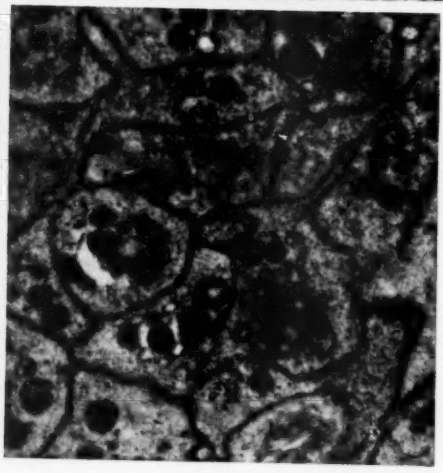
FIG. 13. Leptomeningeal cells of the core are here oriented longitudinally. Their protoplasmic processes may extend for some distance and be fitted together in a complex manner. Mitochondria (m) are moderately abundant. Collagen (cl) is often present between cells. 225 days after operation. × 12,000.

FIG. 14. Leptomeningeal core. Obliquely sectioned cytoplasmic processes are in this view fitted together much like pieces of a jig-saw puzzle. 225 days after operation. × 13,500.

FIG. 15. Leptomeningeal core. The cellular extensions are seen in transverse section. Their manner of apposition is obvious. Small mitochondria (m) are scattered throughout the cytoplasm. 225 days after operation. × 17,000.

13

14



15

EXPERIMENTAL INFECTION OF RHESUS MONKEYS WITH COLORADO TICK FEVER VIRUS*

ROBERT K. GERLOFF, M.A., and CARL L. LARSON, M.D.

From the United States Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Mont.

Since the original isolation of Colorado tick fever (CTF) virus by inoculation of hamsters with human blood,[1] several other experimental hosts have been found. The utilization of suckling white mice has provided a useful means for virus isolations,[2] and the response of the mouse to infection, clinically and pathologically, has been investigated thoroughly.[3] Susceptibility of KB cells from human epidermoid carcinoma, grown in artificial culture, appears comparable to that of suckling mice.[4] Some strains of virus have been adapted to adult mice[5] and to chick embryos.[6]

Natural CTF infection occurs in various rodents which are host to immature stages of the tick *Dermacentor andersoni*, the only known vector of the virus. Infection has been demonstrated[7,8] by isolation of virus from blood or by detection of neutralizing antibody. One rodent, the porcupine *(Erethizon dorsatus)*, can also be infected experimentally, either by intraperitoneal inoculation of virus or by bites of infected adult *D. andersoni*.[9]

In man, CTF infection results in a characteristic diphasic febrile illness, accompanied by marked leukopenia.[8,10] Viremia persists for at least 16 days after the onset of illness. Although death directly attributable to CTF has occurred,[8] it is very rare.

The results of experimental infection of rhesus monkeys are reported here. Attention has been given especially to febrile, hematologic, and serologic responses, duration and intensity of viremia, pathologic lesions, and sites of virus proliferation.

MATERIAL AND METHODS

Preparation of Virus Inoculum

The Florio strain of CTF virus, passed only in hamsters and suckling mice, was used. White mice inoculated intraperitoneally (i.p.) at age 3 days with about 10^4 LD_{50} virus were sacrificed when they became moribund, usually on the fifth day. Ten per cent suspension of their pooled brains was prepared in physiologic saline solution containing 10 per cent normal rabbit serum (NRSS). After light centrifugation,

* Received for publication, January 26, 1959.

the supernate was either inoculated into monkeys immediately or was stored at −70° C. for use later.

Inoculation and Bleeding of Monkeys

Rhesus monkeys, of either sex, each weighing approximately 5 pounds, were used. During any operation, the animal was immobilized, ventral side down, on a small table. Injection of virus was by one of 3 routes: intravenous (i.v.), in the saphenous vein of either leg; intramuscular (i.m.), in the upper thigh region; or subcutaneous (s.c.), in the same area. All blood samples, ranging in size from a single drop to about 30 ml., were taken from the saphenous vein. By application of a tourniquet above the knee, a 21-gauge needle could readily be inserted into the vein. Removal of the tourniquet and gentle backward massage of the vein facilitated withdrawal of blood. If only a drop was needed, the distended vein was merely pricked with the point of a scalpel blade.

Examination of Monkeys and Specimens Therefrom

Febrile Response. All temperatures were taken rectally. Throughout the study, a single thermometer was used for each monkey. Generally 2 temperature determinations were made, the first immediately after the monkey had been secured to the table, the second after about 3 to 5 minutes of immobility. If slight variation between the 2 readings occurred, the average was recorded.

Determination of Viremia. Each blood specimen was homogenized by grinding the clot in the serum with mortar, pestle, and a trace of grinding compound (alundum). Tenfold dilutions of the resulting suspension were made in NRSS, and .05 ml. aliquots of whole blood and of each dilution were inoculated i.p. into six 3-day-old (suckling) mice. Death occurring 3 to 14 days after inoculation was the criterion of infection with CTF virus.

LD_{50} values were calculated by the method of Reed and Muench.[11] In titrations of specimens collected toward the end of viremia, undiluted blood occasionally contained sufficient antibody to neutralize the virus partially or completely, with the result that the specimen was more infectious after dilution than before. The LD_{50} was thus distorted and did not reflect the full amount of virus present. In an effort to compensate for such interference by antibody, the number of mice that undiluted blood probably would have killed in the absence of antibody was estimated. The estimate was based on numbers killed by logarithmic dilutions of the specimen in question and on data from many titrations of blood collected before antibody had developed.

In some instances, the level of virus in the blood was below one LD_{50}, but was sufficient to produce typical symptoms of CTF in one or more mice. Effort was made to recover the virus from the mice by passage of brain tissue from moribund animals to normal suckling mice.

Determination of Pathologic Alterations and Presence of Virus in Tissues. Animals for this purpose were anesthetized with ether and exsanguinated by cardiac puncture. Necropsy was performed, and after a gross pathologic examination, samples of various tissues and organs were removed. Some were placed in formalin or in Orth's fixative solution for sectioning. Other samples were ground in NRSS with mortar, pestle, and alundum, and 10 per cent suspensions (weight/volume basis) were prepared, followed by further 10-fold dilutions. For suppression of the normal bacterial flora on tonsils, it was necessary to add penicillin and streptomycin to suspensions of the tissue. All suspensions were titrated in suckling mice, as previously described.

Hematologic Examinations. Total and differential counts of white blood cells were made by standard methods.[12] On each day of examination, total counts were made in duplicate with 2 separate samples of blood. Differential counts were made in duplicate from a single blood film. All duplicate readings were then averaged. The same person performed all tests for additional uniformity.

Serologic Examination. Blood serums were stored at −20° C. until all specimens from each animal could be tested simultaneously. Tests for neutralizing antibody were performed with cultures of KB cells;[13] titers recorded represent maximum dilution of serum which would neutralize approximately one hundred 50-per-cent infectious doses ($TCID_{50}$) of virus.

Outline of Investigation

Five groups of monkeys, a total of 16 animals, were investigated (Table I). Those in groups 1, 2, and 5 were given comparable massive doses of virus i.v., while those in groups 3 and 4 were given graded doses s.c. for comparison of the susceptibility of monkeys and suckling mice. Febrile response and the development and persistence of viremia were followed in all monkeys. In addition, hematologic changes were followed in groups 2 and 3. One or 2 months after disappearance of viremia from monkeys in groups 1 through 4, each animal was given a massive challenge dose of virus i.v. Further tests for viremia were then performed to determine the immune status of the animal at the time of challenge. When the second viremia, if it occurred, had also

disappeared, the animal thereafter was given periodic booster inoculations for the production of hyperimmune serum. Each inoculation consisted of 1 ml. of mouse brain suspension (containing about 10^8 mouse LD_{50} of virus) mixed with an equal quantity of arlacel-mineral oil adjuvant; inoculations were given at intervals of 1 to 2 months. Serum samples were also collected periodically to trace antibody development.

Monkeys in group 5 were inoculated simultaneously, and one monkey was taken at weekly intervals for pathologic examination and for tests of various organs for virus content.

TABLE I

Infection of Rhesus Monkeys with CTF Virus, and Duration of Viremia

Group	Monkey no.	Dosage of virus (mouse LD_{50})*	Route of inoculation	Viremia following inoculation* Presence or absence of virus in blood on indicated days after inoculation	Maximum titer
1	17	2×10^8	i.v.	**3-4-5-11-18-28-**(45)-46-53†	2.4
2	385	5×10^7	i.v.	**1-2-3-4-5-6-7-9-11-16-24-*36*-**(84)-85-88	3.8
	380	5×10^7	i.v.	**1-2-3-4-5-6-7-9-11-16-24-36-**(84)-85-88	3.4
	593	5×10^7	i.v.	**1-2-3-4-5-6-7-9-11-16-24-*36*-**(84)-85-88	3.6
3	484	8×10^7	s.c.	**1-2-4-6-8-11-*15*-19-26-** (85)-***86-89***	2.5
	865	8×10^6	s.c.	**1-2-4-6-8-11-15-19-26-33-*44*-**(85)-*86*-89	3.0
	386	8×10^5	s.c.	**1-2-4-6-8-11-15-19-26-*33*-** (85)-86-89	3.0
4	363	8×10^4	s.c.	**1-3-5-8-11-14-18-22-29-36-45-**(78)-80-84-91	2.5
	434	8×10^3	s.c.	**1-3-5-8-11-14-18-22-29-36-*45*-**(78)-**80-*84-91***	2.6
	428	8×10^2	s.c.	***1-3-5-8-11-14-18-22-29-36-45-***(78)-**80-*84-91-*** **98-105**	2.4
	431	8×10^1	s.c.	**1-3-5-8-11-14-18-22-29-36-*45-***(78)-80-*84-*91-98	3.5
	432	8×10^0	s.c.	**1-3-5-8-11-14-18-22-29-36-45-**(78)-**80-84-91-98-105-112-121-*128***	3.4
5	395	3×10^9	i.v.	**4-6**	3.7
	396	3×10^9	i.v.	**4-11-13**	3.6
	950	3×10^9	i.v.	**4-11-18-20**	3.5
	698	3×10^9	i.v.	**4-11-18-25-28**	3.5

* All titrations were performed in 3-day-old mice.

† Figures in bold face type: LD_{50} was attained.
Figures in italics: Virus was recovered, but LD_{50} was not attained.
Figures in standard type: Virus was not recovered.
Figures in parentheses indicate day on which i.v. challenge dose of virus was given.

RESULTS

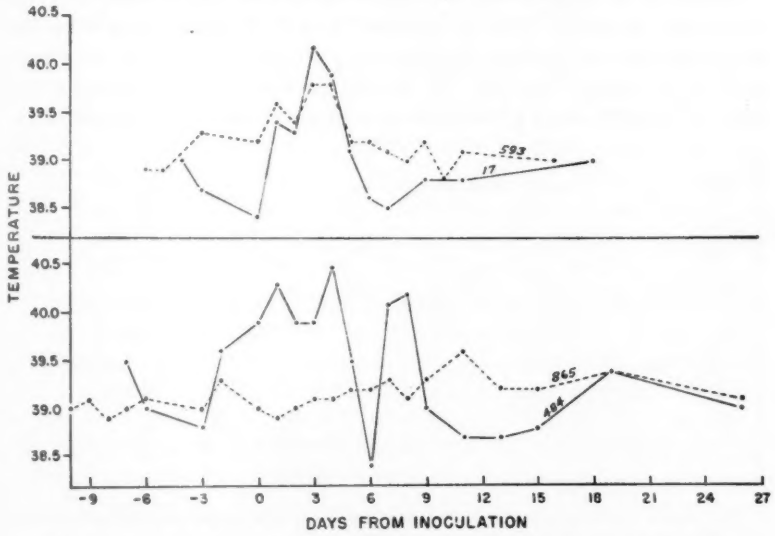
Clinical Response

Easily recognized signs of illness, such as weakness, loss of weight, anorexia, ruffled hair, inactivity, or paralysis were generally absent from these monkeys, regardless of the amount of virus they received or the route of inoculation. Monkey No. 17 was an exception, since it developed a bloody diarrhea on the first day after inoculation; this continued through the third day. The presence of blood may have been caused by slight injury from insertion of the thermometer, but the

diarrhea was not explained. Tests for enteric bacterial pathogens were negative. Although bleeding was not noted in other animals, it could have been associated with CTF infection; a similar condition has been observed occasionally in human cases.[8]

Febrile Response

This also was irregular and undependable as a criterion of infection. Only 3 monkeys (Nos. 17, 593, and 484) developed elevations of temperature which were interpreted as fever (Text-fig. 1). Pre-inoculation temperatures of these and other monkeys usually were between 39.0° C. and 39.5° C. Since extremes of 38.8° C. to 39.9° C. were occasionally observed, only readings of 40.0° C. or above were considered indicative of fever, unless persistent readings definitely above the normal range for a particular animal were obtained.



Text-figure 1. Febrile response of 4 rhesus monkeys to inoculation of CTF virus.

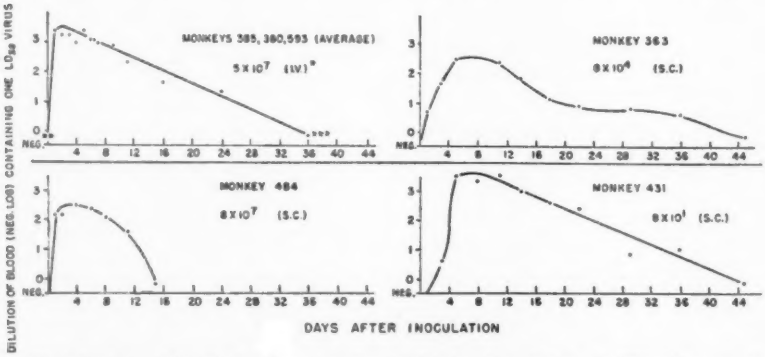
Monkey No. 17 gave the most clear-cut febrile response, with temperature rising above 40° C. on the third day after inoculation and remaining above normal through the fourth day. The pattern of monkey No. 593 was very similar although the maximum temperature attained was lower. Readings above 40° C. were observed more frequently in monkey No. 484 than in any other animal, but its day-to-day temperature was very irregular; at the time of inoculation, it was almost 40° C., and it rose above that level on the first, fourth, seventh,

and eighth days thereafter. The 2 peaks on either side of the very low reading on the sixth day might represent a true diphasic febrile course, similar to that which characterizes human infections. For comparison, the temperature-time curve of monkey No. 865 is also shown. This animal had a longer period of viremia than either Nos. 17 or 593, yet evidence of febrile reaction was lacking. It typifies the other 12 monkeys in this respect.

Viremia and Resulting Immunologic Response

Observations regarding duration and intensity of viremia in all 16 monkeys are summarized in Table I. The shortest period of viremia was 15 days in monkey No. 484; the longest was 50 days in monkey No. 432, after it had been given the challenge dose of virus; the average for all animals in groups 1 through 4 was about 36 days.

Duration of viremia did not appear related to the amount of virus inoculated or to the route of inoculation, but the size of the dose did affect the interval between injection of virus and the earliest detection of it in the blood (Text-fig. 2). A massive dose of virus (between 10^7 and 10^8 mouse LD_{50}) given either i.v. or s.c. resulted in viremia at or



Text-figure 2. Viremia in rhesus monkeys following inoculation of different amounts of CTF virus by two routes.

* Doses of virus are expressed as LD_{50} by titration in 3-day-old mice.

** Neg.: No sign of infection and no virus recovered.

*** Virus was recovered but LD_{50} was not attained.

very near its maximum level on the first day thereafter (monkeys Nos. 385, 380, 593, and 484). With 1,000th of this amount of virus (monkey No. 363), development of maximum viremia was delayed until the fifth day, and with one millionth of this amount (monkey No. 431), virus was undetectable on the first day and still at a very low level on the third. In any monkey, once the titer of virus had

reached its maximum level, it generally remained stable for 5 or 6 days and then gradually decreased.

Monkeys in groups 1 and 2 were solidly immune to the challenge dose of virus; none of these animals developed viremia thereafter (Table I). In groups 3 and 4, this immunity occurred irregularly. Its presence or absence was not related to the amount of virus originally used to infect the monkey or to the length of viremia which followed. It did appear related, however, to the titer of neutralizing antibody in the serum at the time of challenge. Viremia after challenge was of short duration in all monkeys except No. 432 which had not been infected by the initial inoculum.

Results with monkey No. 431 indicate that monkeys are nearly as susceptible to CTF virus as are suckling mice and on a comparable weight basis are more susceptible. No. 431 received 80 mouse LD_{50} of virus and became strongly infected, whereas No. 432 received 8 LD_{50} and resisted infection.

Pathologic Response

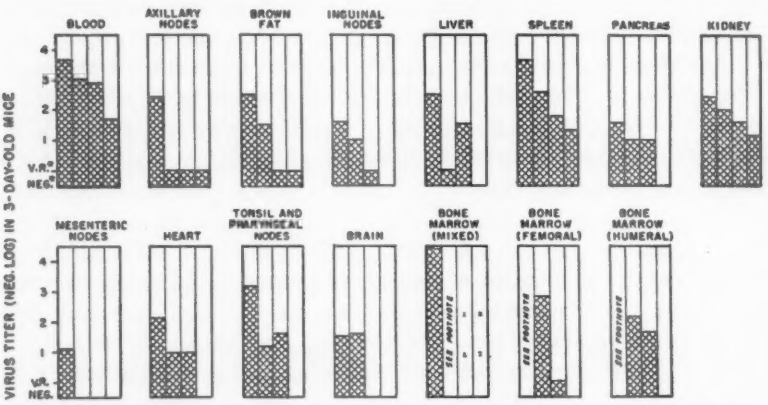
Pathologic examination of monkeys in group 5 did not reveal any particular site of damage by or proliferation of virus. Necropsy was performed on monkeys Nos. 395, 396, 950, and 698 on the sixth, 13th, 20th, and 28th days, respectively, following i.v. inoculation with 3×10^9 mouse LD_{50} of virus, the largest dose used throughout the study. Macroscopic alterations were not detected in any of these monkeys. Portions of the following tissues or organs were sectioned and examined microscopically: kidney, liver, spleen, adrenal gland, lung, heart, pancreas, thymus, small intestine, thyroid gland, sternal bone marrow, abdominal muscle, assorted visceral lymph nodes, cerebral cortex (motor area), midbrain, cerebellum, medulla oblongata, and the cervical and lumbar spinal cord. Prominent, partially hyalinized reaction centers were seen in splenic lymphoid nodules of monkeys Nos. 950 and 698. This lesion, though clearly of a nonspecific nature, may have been related to infection with CTF virus. Histologic alterations were not found in any other tissue examined.

Localization of Virus in Tissues

Titration of virus in various tissues from monkeys in group 5 likewise did not yield conclusive data regarding sites of virus proliferation (Text-fig. 3). On the sixth day after inoculation, virus was abundant in all tissues tested, particularly in bone marrow. Thereafter, it gradually diminished and by the 28th day was not detectable in inguinal,

mesenteric, or pharyngeal nodes, liver, pancreas, heart, tonsil, brain, or bone marrow. Blood consistently contained more virus than any other tissue, and traces of blood occasionally may have accounted for low levels of infectivity in other tissues.

On the 28th day, only in blood, spleen, and kidney could as much as one LD_{50} of virus be demonstrated. Mice inoculated with spleen died sooner than those inoculated with an equal amount of kidney. On the basis of total evidence, our conclusion was that the virus was retained in slightly greater amount by the spleen than by the kidney.



Text-figure 3. Localization of CTF virus in various parts of the rhesus monkey after different intervals post-inoculation.

* V.R.: Virus was recovered but LD_{50} was not attained. Neg.: No sign of infection and virus not recovered.

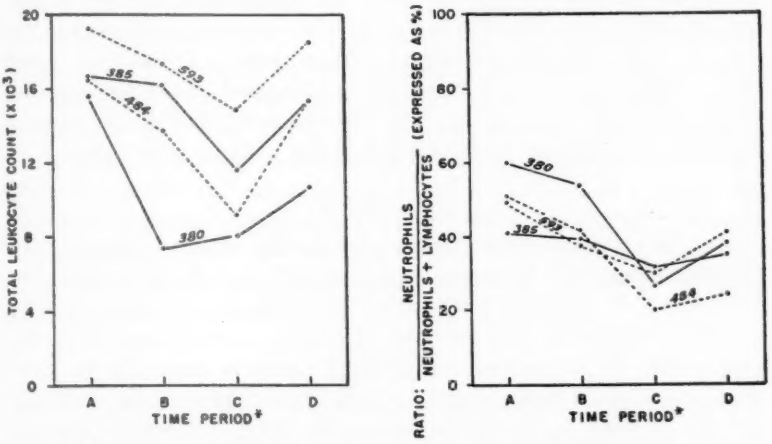
The 4 columns from left to right in each section represent tests done on the sixth, 13th, 20th, and 28th days after inoculation. Femoral and humeral bone marrow were mixed for test on the sixth day but were treated separately thereafter.

Hematologic Response

Examinations of blood for the detection of changes in the total leukocyte count and in the ratio of neutrophils to lymphocytes as a result of CTF infection were performed on the 6 monkeys in groups 2 and 3. Cell counts made on 5 different days preceding inoculation (period A) established the normal cell pattern for each animal. These counts were compared with others made 1, 2, and 3 days (period B); 4, 5, 6, and 7 days (period C); and 9, 11, 16, and 24 days (period D) after inoculation.

The 3 monkeys in group 2 and No. 484 of group 3 received nearly identical amounts of virus. No. 484 differed only in being inoculated s.c. instead of i.v. These 4 monkeys developed a definite leukopenia which began in period B, became most marked in period C, and started

to diminish in period D (Text-fig. 4). Although the total percentage of neutrophils and lymphocytes remained nearly constant (95 to 98 per cent), the proportions of the two types of cells within these totals became markedly different following infection. Development of leukopenia was accompanied by a reduction in the percentage of neutrophils and an increase in the percentage of lymphocytes. Again this change began in period B, was most marked in period C, and began to



Text-figure 4. Variations in the total leukocyte count and the proportion of neutrophils and lymphocytes in 4 rhesus monkeys following infection with CTF virus.

* A: Pre-inoculation days 0, 3, 5, 6, and 7; B: post-inoculation days 1, 2, and 3; C: 4, 5, 6, and 7; and D: 9, 11, 16, and 24.

Readings within each period were averaged. Days for examination of monkey No. 484 were slightly different.

disappear in period D. With regard to reduction of both the total leukocyte count and the percentage of neutrophils, monkey No. 380 was most severely affected; its leukopenia became most marked in period B and remained nearly the same in period C. Monkeys Nos. 865 and 386, which were inoculated at the same time as No. 484 but with less virus, developed only slight, probably not significant, hematologic alterations.

Serologic Response

Because of the blood requirement for other investigations, daily serum samples were not collected from any monkey for follow-up of antibody development immediately after infection. However, the composite serologic data on all 16 animals yielded some information regarding the rise of antibody after a single injection of virus. Of the last 3 monkeys in group 5, one had developed a trace of antibody (1:4 dilution partially neutralized the virus) by the 13th day, although

another still had none by the 20th day; the third monkey had a 1:8 titer on the 15th day and 1:16 on the 28th. Monkey No. 17 (group 1) had 1:128 titer on the 45th day; on the 84th day those in group 2 had titers of 1:128, 1:32, and 1:512.

Each additional injection of virus, up to a total of 4, continued to raise the antibody titer in each animal. Thereafter, further increase was less certain, and in many instances, additional virus had no effect except to maintain the existing titer. The highest titer attained in any animal was 1:4096, which occurred in Nos. 17 and 386 after 11 injections of virus. This was the largest number given to any animal except No. 17 which received 13; however, the additional 2 injections gave no further increase in antibody. The titer in monkey No. 431 after 4 injections was 1:2048; it did not rise higher after 10 injections.

Discussion

The data indicate that criteria of infection must accompany any statement regarding resistance or susceptibility of rhesus monkeys to CTF virus. If clinical or pathologic response is the criterion, monkeys would be considered very resistant; if febrile or hematologic response, they would be considered susceptible irregularly and only to large doses of virus; if lengthy viremia or high levels of neutralizing antibody are the criteria, they would be termed extremely susceptible, even to minute quantities of virus.

The value of monkeys for CTF research would seem very limited, since infection can be detected dependably only through tests for viremia or neutralizing antibody, both relatively laborious procedures. Perhaps the greatest value of monkeys would be for production of hyperimmune serum. Such serum, necessary for identification of viral isolates, is generally prepared by inoculation of hamsters, each of which yields only a small quantity of blood. Monkeys, on the contrary, can be bled and re-inoculated repeatedly. The high neutralizing capacity of immune monkey serum prepared in this investigation was confirmed by tests in mice also; undiluted serum from monkey No. 17, for example, with a titer of 1:4096 in tissue culture tests, neutralized $10^{6.2}$ mouse LD_{50} of virus per 0.1 ml.

The duration of viremia in these monkeys was especially interesting; it was practically identical with that observed in experimentally infected porcupines.[9] The ability of the virus to proliferate and to exist in the blood for long periods of time without apparent harm to the host seems to characterize CTF infection in several animals, including those infected in nature. The persistent viremia probably is important in the maintenance of CTF virus in nature. Present evidence suggests[8]

that infected nymphal *D. andersoni* infect small rodents by feeding on them early in the spring. Later in the season, virus is still present in the blood of these rodents, and larvae of the new generation, in turn, become infected by feeding on them. It is possible, however, that laboratory-adapted strains of virus vary markedly from those in nature with respect to duration of the viremia they induce.

Termination of viremia conceivably occurs as a result of both decreasing virus proliferation and increasing amounts of antibody. In monkeys, the effect of antibody on the intensity of viremia first became apparent on the seventh to 14th days after inoculation, depending on the dosage of virus. Thereafter, the effect steadily increased; whole blood, and sometimes also a 10^{-1} dilution, gradually lost infectivity. For example, whole blood from monkey No. 698 on the 28th day killed none of 6 mice; 10^{-1} dilution killed 6 of 6; 10^{-2} killed 2 of 6; and higher dilutions were again noninfectious. When the period of viremia in the monkeys had nearly ended and the virus had become barely detectable, it was recovered more frequently from 10^{-1} or 10^{-2} dilution than from undiluted blood.

Summary

Response to infection with Colorado tick fever (CTF) virus was investigated in 16 rhesus monkeys. The most dependable early sign of infection was viremia, which persisted for 15 to 50 days, averaging about 36 days. Virus titers in blood reached a maximum of $10^{3.8}$. Clinical symptoms were absent, and a febrile response was observed in only 3 monkeys. Total leukocyte counts and percentages of neutrophils diminished somewhat between the fourth and seventh days after inoculation, although these hematologic alterations seemed dependent on the injection of a massive dose of virus. Neither macroscopic nor microscopic pathologic lesions attributable to CTF infection were observed. Virus was found through the 20th day in a variety of tissues and organs, but on the 28th day it was detected only in kidney and spleen, in addition to blood. Circulating antibody was first detected on the 15th day. Repeated injections of virus beyond the fourth injection had relatively little effect in producing further increases in titer of antibody. The highest titer attained in any monkey was 1:4096, after 11 injections of virus.

REFERENCES

1. Florio, L.; Stewart, M. O., and Mugrage, E. R. The experimental transmission of Colorado tick fever. *J. Exper. Med.*, 1944, **80**, 165–188.
2. Oliphant, J. W., and Tibbs, R. O. Colorado tick fever; isolation of virus strains by inoculation of suckling mice. *Pub. Health Rep.*, 1950, **65**, 521–522.

3. Hadlow, W. J. Histopathologic changes in suckling mice infected with the virus of Colorado tick fever. *J. Infect. Dis.*, 1957, **101**, 158–167.

4. Pickens, E. G., and Luoto, L. Tissue culture studies with Colorado tick fever virus. I. Isolation and propagation of virus in KB cultures. *J. Infect. Dis.*, 1958, **103**, 102–107.

5. Koprowski, H., and Cox, H. R. Colorado tick fever. I. Studies on mouse brain adapted virus. *J. Immunol.*, 1947, **57**, 239–253.

6. Koprowski, H., and Cox, H. R. Colorado tick fever. II. Studies on chick embryo adapted virus. *J. Immunol.*, 1947, **57**, 255–262.

7. Eklund, C. M.; Kohls, G. M., and Jellison, W. L. Isolation of Colorado tick fever virus from rodents in Colorado. *Science*, 1958, **128**, 413.

8. Eklund, C. M.; Kohls, G. M.; Jellison, W. L.; Burgdorfer, W.; Thomas, L. A., and Kennedy, R. C. The Clinical and Ecological Aspects of Colorado Tick Fever. Presented at the International Congress on Tropical Medicine and Malaria, Lisbon, Portugal, 1958.

9. Burgdorfer, W. Colorado tick fever. The behavior of CTF virus in the porcupine. *J. Infect. Dis.*, 1959, **104**, 101–104.

10. Cox, H. R. Colorado Tick Fever. In: Viral and Rickettsial Infections of Man. Rivers, T. M. (ed.) J. B. Lippincott Co., Philadelphia, 1952, ed. 2, pp. 526–530.

11. Reed, L. J., and Muench, H. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, 1938, **27**, 493–497.

12. Kolmer, J. A., and Boerner, F. Approved Laboratory Technic. D. Appleton-Century Co., New York, 1945, ed. 4, pp. 66–92.

13. Gerloff, R. K., and Eklund, C. M. A tissue culture neutralization test for Colorado tick fever antibody and use of the test for serologic surveys. *J. Infect. Dis.*, 1959, **104**, 174–183.

We wish to thank Dr. William J. Hadlow, formerly of this Laboratory, for performing all histologic examinations; also Mr. Rex O. Watson, Mrs. Monterey B. Leach, and Miss Lillian R. B. Glesne for technical assistance throughout the study.

EXPERIMENTAL INHALATION ANTHRAX IN THE CHIMPANZEE *

WILHELM S. ALBRINK, Ph.D., M.D.,† and ROBERT J. GOODLOW, Ph.D.
From the U.S. Army Chemical Corps, Fort Detrick, Frederick, Md.

Although anthrax has long been recognized in domestic animals and man, precise techniques have only recently been devised to allow a controlled study of the disease in experimental animals by utilizing a respiratory route of infection.[1] "Woolsorters' disease" and "ragpickers' disease" have been described in man, and a portal of entry by way of the tracheal or bronchial mucosa has been suggested as a basis for the subsequent development of "anthrax pneumonia."[2,3] Another interpretation of the observations in human cases suggests that the lung parenchyma serves as a portal of entry for organisms and that a generalized systemic infection rather than a primary pneumonic process results.[4] Experiments using a variety of animals have tended to support the latter view,[5,6] and recent investigation in guinea pigs has demonstrated the passage of spores from the alveolar spaces to regional lymph nodes with subsequent germination and dissemination of the vegetative forms.[7]

The experiments reported here were designed to correlate the clinical behavior of infected animals with the development of the disease and to determine the tissue alterations in higher primates, with particular reference to the respiratory tree.

MATERIAL AND METHODS

The experimental animals consisted of 4 young, healthy chimpanzees, weighing from 18 to 23 pounds. All but one were classified as *Pan troglodytes* (*Schwarz*); the exception (Bill) belonged to the group *Pan troglodytes troglodytes*. The animals had complete pre-exposure physical examinations, and records were made of blood counts, blood cultures, stool examinations, rectal temperatures and roentgenograms of the chest. All reports indicated that the animals were normal except that occasional hookworm ova were encountered in the stools. Following exposure, the chimpanzees were examined daily, and the blood was cultured. Rectal temperature was taken twice daily, and chest roentgenograms were obtained every other day.

Five 10-fold dilutions in saline were made of each sample of blood. Three plates each of cysteine blood agar and Wilson's peptone agar

* Received for publication, January 7, 1959.
† Present address: Department of Pathology, Yale University School of Medicine, New Haven, Conn.

were inoculated with 0.1 ml. of each dilution of blood as well as undiluted blood. The plates were incubated for 24 hours at 37° C., after which colonies were counted. In cases where no colonies were observed at the first reading, the plates were re-incubated for 24 additional hours and then reread. Each of 5 mice received intraperitoneal injections (0.5 ml.) of the first 1:10 dilution of each blood sample. When the mice died, heart blood smears and culture on agar plates substantiated the presence of *Bacillus anthracis*.

The strain of *B. anthracis* used was designated Vollum 1B. The concentrations of spores in the generator were adjusted to be within the limits of 4×10^6 and 6×10^6 spores per ml. of suspension medium (casein acid digest containing one per cent phenol).

Aerosol was generated by means of a University of Chicago Laboratory (UCTL) atomizer, introduced into a modified Reyniers chamber[8] and maintained at a temperature of 25° C. ± 1 degree and a relative humidity of 30 ± 3 per cent. The UCTL atomizer was operated with a total air flow of 100 liters per minute (5 liters of primary air at a pressure of 12 pounds per square inch and 95 liters of secondary or mixing air), and the liquid suspension of *B. anthracis* spores was disseminated from the atomizer by means of a constant feed device at a rate of approximately 0.2 ml. per minute. The aerosol was continuously generated directly into a 120-liter mixing chamber where it was kept turbulent, mixed, and at a constant spore concentration.

At the time of animal exposure, the aerosol from the mixing chamber was drawn through a 16-inch length of 2-inch diameter tubing into a mask fitted tightly to the face of the chimpanzee by a pneumatic gasket. The aerosol flowed over the face of the animal at a rate of 8.7 liters per minute. The chimpanzee, anesthetized with Nembutal,® inhaled the cloud for 5 minutes. A critical-orifice, liquid impinger with a flow rate of 2.5 liters per minute was employed to determine the viable spore concentration of the aerosol. This impinger was situated at a point in the mask directly over the nostrils of the animal and was operated continuously during the entire exposure period. Since the sample was drawn from a cloud at exactly the same level as the chimpanzee's face, it represented the number of viable spores available for inhalation per unit volume (liter) of aerosol.

After exposure, the collecting fluid (tryptase saline) of the impinger was suitably diluted and plated on Wilson's peptone agar. By knowing the volume of aerosol sampled and by determining from plate counts the concentration of viable spores in the collecting fluid, it was possible to calculate the number of viable spores per liter of aerosol. Prior to exposure, the volume of air inhaled by the animal was estimated by a

direct measurement with a dry test meter. Also, as a check on the direct measurement of inhaled volume, an additional estimate was made during the actual exposure by measuring the amplitude recorded on a Sanborn recorder connected to a strain gauge which had been calibrated with a dry test meter immediately prior to the experiment. The strain gauge was taped to the sternum of the chimpanzee. In this way a reasonably reliable estimate of the minute-volume was achieved, and the strain gauge measurements could be used as a continuous check of the respiratory volume while the animal was in the exposure apparatus and direct measurements were impossible. The total number of viable spores inhaled by the chimpanzee was calculated by multiplying the number of viable spores in a liter of aerosol by the minute-volume (liters) and the duration of exposure (minutes).

Direct microscopic measurements were made on aerosol particles collected by means of a millipore filter and on glass slides in a settlement chamber. The number median diameter of these particles collected at the face mask was about 1.3 μ and the volume median diameter, 8.5 μ.

Results

Clinical Observations

The pertinent data are summarized in Table I.

The first two animals (John and Melvin) exhibited no positive physical disorder after their initial exposure and survived despite the fact that organisms were demonstrated in the blood of one on the second through the tenth days and of the other from the third through the eleventh days. The animals maintained their appetites and their vigorous protestations to physical examination in unabated manner. Although a bacteremia was apparent in each, it was of low grade and exhibited no progression. The temperature varied little from normal (100° to 101° F.).

In the second pair of animals exposed (Bill and Grove), blood cultures were positive for *B. anthracis* on the second and third days, and the bacteremia progressed rapidly as determined by plate counts. No abnormalities of behavior and no abnormalities in physical examination were apparent until the morning of the day of death. At that time, both animals exhibited marked tenderness on palpation in the region of the spleen, an observation which represented the first physical sign of illness in either. They were observed at 4:00 p.m., and both seemed to be normal to casual inspection at that time. When next checked at 8:00 p.m., one (Bill) was dead, and 2 hours later the other (Grove) was noted to be in some distress. A careful physical examination was performed. Temperature was subnormal (97.8° F.); the animal appeared

TABLE I

Colonies of B. anthracis in the Blood, and Mortality Rate in Challenged Mice on Successive Days after Exposure

	First exposure				Second exposure	
	Melvin	John	Grove	Bill	Melvin	John
Dry test meter dose	32,800	34,350	39,700	66,500	90,300	112,000
Aerosol, median particle diameter (μ)	1.4	1.4	1.2	1.05	1.4	1.4
Fate of chimpanzee	Survived	Survived	Died	Died	Survived	Died
Days after exposure:						
First						
Colonies*	0	0	—	0	0	0
Mort. in mice†	—‡	—	0/5	1/5	0/5	0/5
Second						
Colonies	3	—	3	3	0	0
Mort. in mice	—	—	1/5	1/5	0/5	0/5
Third						
Colonies	30	18	43	70	0	0
Mort. in mice	—	—	5/5	2/5	0/5	0/5
Fourth						
Colonies	18	13	380	506	23	68
Mort. in mice	—	—	5/5	5/5	0/5	4/5
Fifth						
Colonies	—	12	810	660	40	1,630
Mort. in mice	—	—	5/5	5/5	1/5	5/5
Sixth						
Colonies	0	0	13,000 117,000§	90,300	0	13,500
Mort. in mice	4/5	5/5	5/5	5/5	0/5	5/5
Seventh						
Colonies	0	13			0	2.89×10^3
Mort. in mice	—	—			0/5	5/5
Eighth						
Colonies	6	10			0	4.3×10^3 (at necropsy)
Mort. in mice	5/5	5/5			0/5	5/5
Ninth						
Colonies	—	—				
Mort. in mice	—	—				
Tenth						
Colonies	0	0				
Mort. in mice	3/5	3/5				
Eleventh						
Colonies	0	0				
Mort. in mice	—‖	2/5‖				

* Colonies: Number of colonies per ml. of blood.
† Mort. in mice: Mortality rate in challenged mice.
‡ Minus sign indicates test not done.
§ One and a half hours before death.
‖ Negative on subsequent tests.

lethargic and was hard to arouse. His lips and mucous membranes were cyanotic, the pulse was rapid and weak, and respirations were shallow and labored. Tubular breath sounds were apparent on auscultation, and percussion of the chest yielded dullness. He expired 1½ hours later.

The 2 animals which survived initial exposure were rechallenged with a larger dose approximately 6 weeks after the blood cultures became negative. Organisms were demonstrable in the blood of Melvin on the fourth and fifth post-exposure days, but he exhibited no symptoms and thereafter maintained a negative blood culture and had an uneventful recovery. John also developed a positive blood culture on the fourth post-exposure day, and at this time exhibited splenic tenderness. His blood, however, showed a rapidly increasing bacteremia, and he died early on the eighth day after re-exposure. Examination approximately 4 hours before death revealed a temperature of 97° F. and an enlarged spleen, although the overall clinical impression was one of marked improvement.

NECROPSY EXAMINATION

Gross Observations

Bill. Each pleural cavity contained approximately 500 ml. of straw-colored, slightly turbid fluid. The pericardial and peritoneal cavities contained a slight excess of similar fluid. The mediastinal adipose and connective tissues were incorporated into a translucent, edematous mass of gelatinous consistency. The parietal pleura, particularly on the right side, had a similar appearance. The visceral pleura over most of both lungs was thickened with edema fluid. The lungs were mottled pink and dark red in color. When the pulmonary substance was cut, frothy fluid emerged from the pink area, and the dark red regions were hemorrhagic and airless. The trachea and major bronchi contained frothy pink fluid, and the mucosa contained small bright red hemorrhages but no erosions or ulcerations. The lymph nodes on the posterior aspect of the tracheal bifurcation were greatly enlarged and hemorrhagic, with complete loss of grossly recognizable architecture (Fig. 1). This mass of nodes was connected to a smaller, less hemorrhagic group adjacent to the ascending limb of the aortic arch by means of a dark red, beaded lymphatic vessel (Fig. 2). There was a similar enlarged hemorrhagic node adjacent to the thymus, and comparable single nodes were found in the pancreatic chain, as well as in a group draining a dilated loop of duodenum covered by congested but not hemorrhagic serosa. The spleen was enlarged and soft, and its cut surface appeared uniformly dark red in color. The liver was markedly congested. The

meningeal vessels were congested, and the brain was edematous, but neither hemorrhage nor exudate were noted. The heart, pancreas, kidneys, adrenals, testis, thyroid, pituitary and urinary bladder showed no gross lesions. The colon at the hepatic flexure exhibited a large abscess attributable to a parasitic worm. Except for this and the dilated loop of duodenum mentioned previously, there were no gross lesions of the gastrointestinal tract.

Grove. The gross observations were qualitatively similar to those described for Bill.

John. Although this animal exhibited the large soft spleen and gelatinous mediastinal edema seen in the first two, he did not have pleural effusion, hemorrhagic tracheobronchial lymph nodes or massive pulmonary edema and hemorrhage. Edema of the tracheal mucosa was prominent, but no hemorrhage was evident. The only extensive hemorrhages were found in the cortex and medulla of both adrenal glands.

Microscopic Observations

The first two animals which succumbed (Bill and Grove) presented essentially similar histologic alterations, differing only in degree of involvement. The extent and severity substantiated the impressions derived from the gross observations. Since Bill presented the more accentuated changes, the lesions in him will be described. The pleura was thickened as the result of separation of the connective tissues by edema fluid devoid of vegetative anthrax bacilli or cellular exudate (Fig. 3). The pulmonary vessels were markedly distended with blood and a myriad of anthrax bacilli (Fig. 4). The alveoli contained edema fluid in some regions and were filled with blood and bacilli in others. Scanty cellular exudate and few bacilli were found in the alveoli free of hemorrhage. The bronchioles and small bronchi contained variable quantities of blood, mucus, and organisms. The enlarged, congested lymph nodes at the tracheal bifurcation and adjacent to the thymus, pancreas, and duodenum, exhibited almost complete replacement of recognizable architecture by massive hemorrhage (Fig. 5) and accumulations of neutrophils. Elsewhere, the nodes showed marked dilatation of sinusoids, a dearth of lymphocytes, and small, follicular, germinal centers. Some of the latter were necrotic. The spleen retained recognizable architecture, but germinal centers were surrounded by zones of hemorrhage and many organisms were distributed throughout (Fig. 6). The liver was not remarkable except for extreme acute passive congestion and numerous bacilli in the sinusoids. The blood vessels of the kidneys were congested and contained many bacilli. Some of the glomerular spaces and many of the proximal convoluted tubules con-

tained granular, pink-staining, noncellular substance. The proximal tubules were slightly dilated, and the tubular epithelium was somewhat swollen. The intestine of the worm found in the colonic abscess contained organisms morphologically similar to *B. anthracis*. The dilated loop of duodenum mentioned in the gross description was the seat of serosal congestion, but no other pathologic alterations were noted. The gelatinous swelling of fat and connective tissue observed in the mediastinum of all 3 animals and about the kidneys and adrenals of Grove and John revealed edema but little cellular exudation or hemorrhage. The other tissues examined (heart, pancreas, adrenal, testis, urinary bladder, thyroid, tonsil, pituitary, and brain) contained anthrax bacilli in blood vessels but no lesions.

The animal dying following a second exposure (John) exhibited few histologic lesions. The lungs showed minimal edema and focal congestion. The tracheal and bronchial mucosa were edematous, but no cellular exudate was apparent. The spleen resembled the spleens of the other two animals. Some of the lymph nodes contained dilated sinusoids and exhibited a paucity of lymphocytes, but the architecture was maintained, and there was no massive hemorrhage. The mediastinal and perirenal tissues were identical in histologic appearance to their counterparts in the other animals. There was hemorrhage in the cortex and medulla of both adrenal glands. No other significant microscopic features were observed.

Discussion

These experiments indicate that the chimpanzee is susceptible to *B. anthracis* when it is administered in aerosols of small particle size via the respiratory route. At a calculated inhaled dose of 32,000 to 66,500 spores, bacteremia appeared on the second or third day following exposure, and 2 of the 4 animals succumbed on the sixth day. It is noteworthy that in the presence of a bacteremia of considerable magnitude, the 2 animals that died exhibited no objective findings, and until a few hours before death the only evidence of illness was tenderness in the splenic region. The rapidity with which death ensued in apparently healthy animals was dramatic.

In the absence of therapy, the development of positive blood cultures following infection with *B. anthracis* by any route has been considered a grave prognostic sign. It is of interest that the 2 survivors developed a bacteremia on the second or third day following exposure. The bacteremia continued at a low level for 8 days. It is of further interest that one of these animals, on re-exposure to a larger inoculum, developed a delayed bacteremia on the fourth day which persisted for only 2 days and was followed by complete recovery of the animal. It

would appear that the presence of a bacteremia was not necessarily significant as to outcome. A rapid progression in numbers of organisms in the blood, on the other hand, is ominous.

The pathologic observations at necropsy resembled those previously described in the guinea pig, mouse, and monkey. These were characterized by widespread edema and hemorrhage, most prominent in the spleen, lungs, and lymph nodes, and by the absence of a pronounced cellular inflammatory exudate in the lungs. This confirms the impressions of others that *B. anthracis* introduced by the respiratory route into susceptible animals results in a fulminating septicemia rather than a primary pulmonary infection. No tracheal or bronchial lesions comparable to those described in "woolsorters' disease" in man were observed.

The fact that the intraperitoneal injection of blood in mice was followed by death of the animal and cultural recovery of the organism, whereas the inoculation of conventional cultures failed to show growth, indicates that mouse injection is a valuable ancillary diagnostic technique.

It is tempting to speculate on the development of a degree of resistance in the previously exposed animal. This may have accounted for the delay in developing bacteremia on re-exposure, the greater number of organisms in the blood before death, and the less severe edema and hemorrhage observed at necropsy. Although these features were striking, the experience with so few animals is far too limited to permit conclusions.

Summary

Four chimpanzees were exposed to *B. anthracis* via the respiratory route, utilizing small particulate aerosols. Each developed bacteremia within 2 to 3 days of exposure. Two animals survived and 2 died. On re-exposure to larger doses, both survivors of the first experiment developed delayed bacteremia, and one died. The clinical course of the disease was not discernible except by the occurrence of bacteremia.

Necropsy observations were comparable to those described for other relatively susceptible laboratory animals. They indicated that *B. anthracis* administered by the respiratory route caused a fulminating septicemia rather than a localized pulmonary disease.

REFERENCES

1. Druett, H. A.; Henderson, D. W.; Packman, L., and Peacock, S. Studies on respiratory infection. I. The influence of particle size on respiratory infection with anthrax spores. *J. Hyg.*, 1953, 51, 359–371.
2. Greenfield, W. S. Supplementary Report on the Woolsorter's Disease in the Bradford District. Eleventh Annual Report of the Medical Office of the Local Government Board. London, 1881–1882, pp. 207–238.

3. Fraenkel, E. Über Inhalationsmilzbrand. *Virchows Arch. path. Anat.*, 1925, **254**, 363–378.
4. Eppinger, H. Die Hadernkrankheit, eine typische Inhalations-Milzbrandinfection beim Menschen unter besonderer Berücksichtigung ihrer pathologischen Anatomie und Pathogenesis auf Grund eigener Beobachtugen dargestellt. G. Fischer, Jena, 1894, pp. 139–141.
5. Barnes, J. M. The development of anthrax following the administration of spores by inhalation. *Brit. J. Exper. Path.*, 1947, **28**, 385–394.
6. Young, G. A., Jr.; Zelle, M. R., and Lincoln, R. E. Respiratory pathogenicity of *Bacillus anthracis* spores. I. Methods of study and observation on pathogenesis. *J. Infect. Dis.*, 1946, **79**, 233–246.
7. Ross, J. M. The pathogenesis of anthrax following the administration of spores by the respiratory route. *J. Path. & Bact.*, 1957, **73**, 485–494.
8. Rosebury, T. Experimental Air-borne Infection. Williams & Wilkins Co., Baltimore, 1947, 222 pp.

[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Posterior view of chimpanzee's (Bill) lungs, illustrating pulmonary hemorrhages and large hemorrhagic mediastinal lymph nodes. (U.S. Army photograph.)

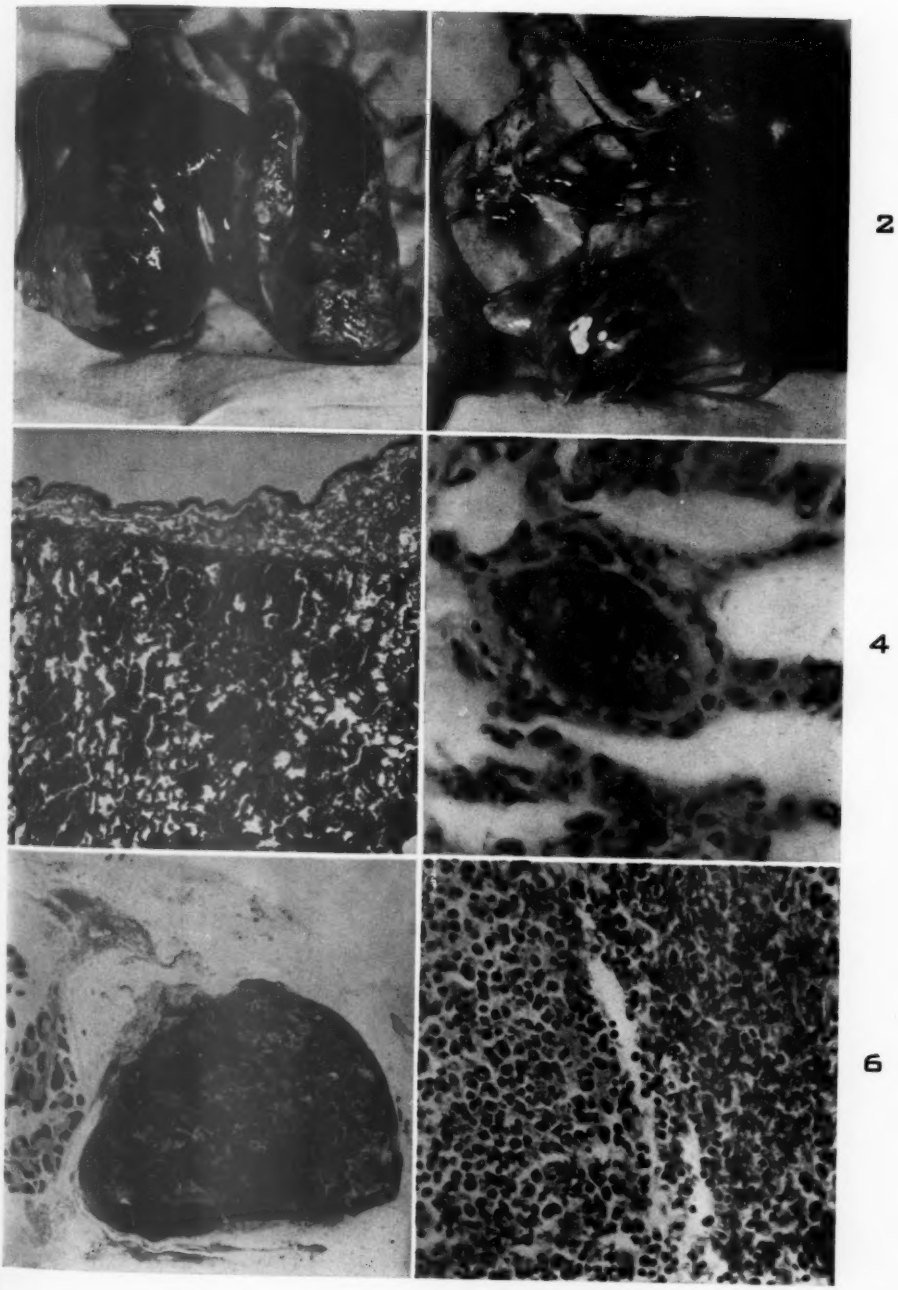
FIG. 2. Posterior oblique view of chimpanzee's (Bill) lungs, demonstrating a markedly dilated beaded lymphatic vessel connecting hemorrhagic nodes on the right with smaller ones on the left. (U.S. Army photograph.)

FIG. 3. Section through chimpanzee's (Bill) lung, illustrating the edematous pleura and hemorrhage into alveolar spaces. Giemsa stain. × 42.5.

FIG. 4. An area of lung (Bill) devoid of hemorrhage, showing partial collapse and congested blood vessels containing many *B. anthracis* organisms. Giemsa stain. × 460.

FIG. 5. Lymph node with architecture essentially obliterated by hemorrhage. The connective tissue about the adjacent thymus is markedly edematous. Giemsa stain. × 6.5.

FIG. 6. Lymphoid nodule of the spleen with necrosis of cells in germinal center and surrounding hemorrhage containing many *B. anthracis*. Giemsa stain. × 290.


1
2
3
4
5
6

CELL DEATH

IV. The Effect of Injury on the Entrance of Vital Dye in Ehrlich Tumor Cells*

D. W. King, M.D.†; S. R. Paulson, M.S.‡; N. L. Puckett, B.S., and A. T. Krebs, Ph.D.
From the Radiobiology Department, Army Medical Research Laboratory, Fort Knox, Ky.

In 3 preceding papers[1-3] the changes occurring in injured Ehrlich tumor cells have been discussed. In this final paper the use of a vital stain, trypan blue, and its relative importance as an indication of the presence of dead and dying cells will be described. In addition, the general pattern of death of cells will be discussed. Although Lepeschkin and Goldman[4] emphasized the early degenerative changes in cellular protein, Rahn[5] demonstrated that coagulation of protoplasm was the very terminal event in cell death. We believe Lepeschkin and Goldman's observations were indicative of coagulative conglomeration of protein following rupture of cell membranes and had no relation to the early unfolding or denaturation of the globular proteins, a phenomenon which occurs much earlier in the degenerative process.

Vital dyes have long been used to determine cell viability. It is an old dictum of biologists that dyes will never enter the living nucleus.[6] Rahn used methylene blue as an indicator of alterations in cellular permeability and found that injured cells took up the dye long after they had lost the capacity to divide and to ferment glucose. However, methylene blue undoubtedly enters the cell and is present in a reduced form long before it can be seen.

Evans and Schulemann[7] concluded that the entrance of trypan blue into the cell was purely a physical phenomenon based on the size of the particle and was not due to the chemical union between dye and protoplasm. In experiments with Ehrlich tumor cells, it proved to be an excellent guide as to both structural and metabolic activity but a poor indicator of changes in cellular permeability. Marked alteration in the amount of cellular protein, sodium, potassium and water occurred before the dye was taken up by the cell.

Experiments

The incubation experiments were carried out as described in the previous papers.[1-3] Cells were incubated with .0014 M glucose or

* Received for publication, August 14, 1958.

† Present address: Department of Pathology, Yale University School of Medicine, New Haven, Conn.

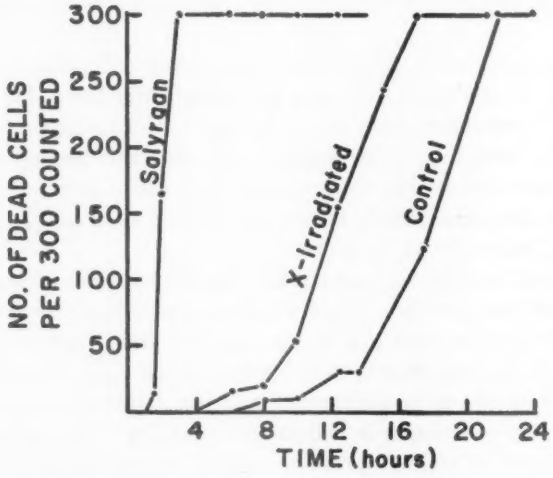
‡ Present address: Department of Zoology, University of Wisconsin, Madison, Wis.

pyruvate, maintained under air or nitrogen and irradiated with 1×10^6 r.

An aqueous solution (0.01 per cent) of trypan blue was used as a vital stain. One drop was added to 3 drops of cell suspension under the cover slip of a standard blood counting chamber. Three hundred to 500 cells were counted at each time period, and the percentage of cells taking the stain into the nucleus was noted.

Results

Trypan blue was used as a test for the viability of Ehrlich tumor cells. The entrance of trypan blue into the cell occurred later than the derangement of the other processes measured (Text-fig. 1). One hundred per cent of the salyrgan-treated cells usually completed the uptake of trypan blue in less than one hour. The irradiated and control cells had similar curves at a later hour. In many experiments of 6 to 8 hours duration, the control and irradiated cells never took up trypan blue.



Text-figure 1. The effect of x-irradiation (1×10^6 r.) and salyrgan (0.001 M) on the viability of Ehrlich tumor cells (stained with trypan blue) in a typical experiment.

Following rupture of the cell as the cytoplasmic and nuclear protein gradually dissolved in the surrounding medium, the dye became lighter and lighter until the final remnants failed to stain.

There were, however, histologic alterations noted in the cell structure before the entrance of trypan blue and the subsequent rupture of the cell. M. Lewis[8] found a brightening and accentuation of the nuclear membrane in tissue culture fibroblasts. In Ehrlich tumor cells,

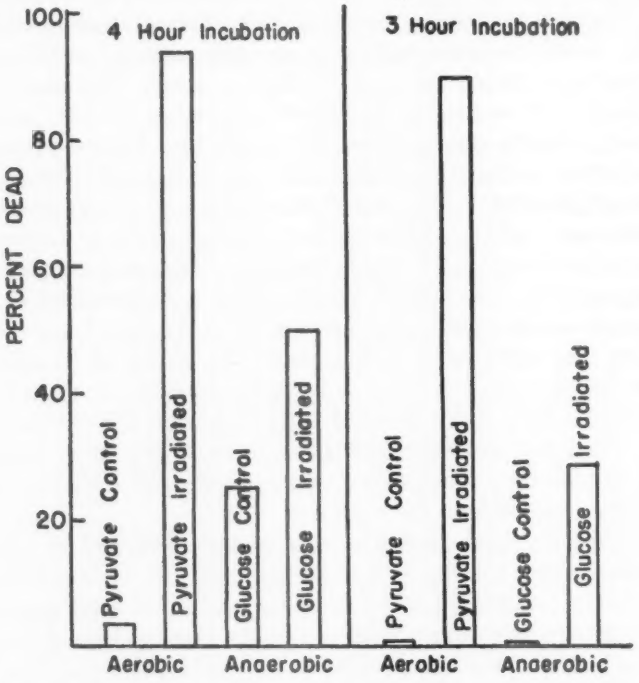
this occurred principally after the shrinkage of the nucleus and before rupture of the cytoplasmic membrane.

The formation of cytoplasmic blebs similar to pseudopods has been seen regularly in tissue cultures.[9,10] Although they were occasionally observed in control cells, they were found principally in the irradiated cells in our experiments (Figs. 3 and 4). The salyrgan-treated cells usually swelled regularly and homogeneously without exhibiting these localized irregularities in outline (Figs. 5 to 10). The blebs in the irradiated cells sometimes appeared to be clear, in the nature of vacuoles. Protein was not apparent in the fluid in hematoxylin and eosin stained sections. The blebs could represent localized weaknesses in the cell membrane. In other instances, they were opaque and continuous with cytoplasmic structure. Cloudy swelling, a term first used by Virchow, has long been thought to represent an early stage of degeneration. The cells in this condition were described as being large and cloudy, showing fine granularity of the cytoplasm. Virchow thought that injury to the cell impaired the ability of its cytoplasm to assimilate protein brought to it by the blood.[11] In recent years, pathologists have justly attached little diagnostic significance to this term. It is impossible to distinguish between late pre-mortem and early post-mortem alterations. Injury to the cell obviously occurs following removal from the organism.

A similar but more marked process has been described by a number of investigators working with tissue cultures. W. H. Lewis[12] found that the first indication of death of the cell was a hazy granulation of the nucleus and later granulation in the clear peripheral processes of fibroblasts. Later the cytoplasm and nucleus became filled with fine, white granules which at first showed brownian movement. Since they were separate and distinct from the mitochondria, they may have been conglomerate, denatured, corpuscular proteins. Present workers in tissue culture have described this latter phenomenon.[13] Photographs taken in this laboratory of unfixed Ehrlich tumor cells injured with salyrgan and roentgen rays (Figs. 5 to 10) show the typical features of cloudy swelling.

Trypan blue was also of value in attempts to measure the effect of injury on the aerobic and anaerobic parts of the glucolytic cycle. Heinmets and Kathan[14] thought from their studies with bacteria that the fermentative cycle was more affected than the aerobic cycle. In experiments in this laboratory glucose and pyruvate were added to two aliquots of the same sample of cells to a final concentration of .0014 M respectively. They each received equal doses of irradiation (1×10^6 r.) simultaneously under aerobic conditions. Examination

of the cells by vital stain techniques (trypan blue) showed that the control cells incubated in pyruvate under aerobic conditions and glucose under anaerobic conditions maintained their integrity without damage (Text-fig. 2). However, cells irradiated in pyruvate and



Text-figure 2. The effect of irradiation (1×10^6 r.) on cells incubated under anaerobic and aerobic conditions as measured by the entrance of trypan blue into the cell.

maintained under aerobic conditions had a 90 per cent uptake of vital stain in 3 hours, while only 29 per cent of those irradiated in glucose and maintained under anaerobic conditions took up trypan blue. In a later experiment, 4 hours in duration, the control pyruvate-incubated cells again maintained 100 per cent viability as measured by this technique, and the glucose control cells had 97 per cent viability. The pyruvate-irradiated cells showed evidence of much more damage than the glucose-irradiated cells. In the former group 98 per cent took the stain, while in the latter only 49 per cent did so (Text-fig. 2).

In earlier experiments with two groups of cells not irradiated but given equal amounts of glucose and studied under anaerobic or aerobic conditions, it was shown that the cells in the anaerobic group were unable to maintain the proper electrolyte concentrations, even though

they did not take up vital stain. Control cells given pyruvate maintained normal contents of sodium, potassium, and water. Control cells given glucose and incubated anaerobically failed to maintain normal sodium and potassium levels, although appreciable numbers did not take in vital stain. Irradiated cells given glucose did less well than the two control groups, but did appreciably better in maintaining normal sodium and potassium levels than the cells irradiated in pyruvate.

Thus, from evidence gained by the entrance of vital stain, the maintenance of proper sodium and potassium values, and from the cessation of respiration while lactic acid production continued, it is concluded that in these cells one enzyme or a group of enzymes in the oxidative cycle is more sensitive to radiation in the presence of substrate than the enzymes in the fermentative part of the cycle. Although both groups were irradiated in air, it might be postulated that hydrogen peroxide formed in sufficient quantities would be effective over a longer time period in continually aerated solutions than in solutions kept under anaerobic conditions.

Discussion

The purpose of this investigation was to follow the processes leading to death of a cell and to determine, if possible, which physiologic features of the cell were most susceptible to injury. The results of many experiments on the metabolic processes of degenerating control, irradiated, and salyrgan-treated cells indicated that the sequence of events in Ehrlich tumor cells followed a typical pattern for all 3 forms of injury. This may be divided into 4 stages (Table I).

Few investigators would maintain that the nucleus was essential for short term survival of cells. Red blood cells maintain an active metabolism for 120 days without benefit of a nucleus, and the functions of the nucleus are not well defined.[15] Many investigators have believed that the nucleus was the center of protein synthesis, and Gale and Folkes[16] have demonstrated the importance of deoxyribonucleic acid (DNA) on amino acid incorporation and the formation of adaptive enzymes. However, Brachet and Chantrenne[17] showed that the incorporation of amino acid and C^{14} in the giant alga (*Acetabularia mediterrania*) was not stopped when the cells were enucleated, and Malkin[18] observed similar results with sea urchin eggs. It appears likely that the nucleus will prove important in the general metabolism of the cell although it was no surprise to workers in this laboratory to find that some constituents of the nucleus could be badly damaged without untoward effects on other aspects of the cell's metabolism.

The nucleus became granular, hazy, swollen, and finally pyknotic earlier than its associated cytoplasmic component. It (the nucleus) lost appreciable amounts of DNA, and the mitotic index dropped precipitously before there was any demonstrable alteration in mito-

TABLE I

Stages of Cell Death

Stage 1
1. Division stops, as the cell fails to undergo mitosis.
2. Deoxyribonucleic acid is lost from the cell nucleus.
3. Protein is lost concurrently with the first two steps, probably from both the nucleus and the cytoplasm.
4. The formation of blebs, granules, and minor changes in the size of the nucleus and cytoplasm occur.
Stage 2
5. Respiration ceases several hours later.
6. Lactic acid production continues for a short time following cessation of respiration.
7. Total dehydrogenase activity is lost, with interruption of complex oxidative and fermentative cycles.
8. Mitochondria decrease in number and increase in size throughout the experiment, but they do not disappear completely with the loss of metabolic functions.
Stage 3
9. Potassium loss from the cell is concurrent with the entrance of sodium and water. Although small variations in these constituents may occur earlier, the terminal changes appear to be associated with the loss of energy production following breakdown of the glucolytic cycle.
10. Trypan blue enters the cell and stains the nucleus and cytoplasm.
11. At a critical point, in a rather narrow range, the cell bursts, releasing large amounts of sodium and water. This results in contraction in size, although a protein framework in the cytoplasm and nucleus remains intact.
Stage 4
12. The denatured protein of the cytoplasm takes up additional amounts of sodium and water. Later, as the protein is dissolved into the surrounding medium, the total content of sodium and water is reduced.
13. The terminal picture is that of a few irregularly stained nuclei embedded in a conglomerate mass of precipitated protein.

chondrial structure, plasma membrane structure, amount of ions or water, dehydrogenase activity, respiration or lactic acid production. The only concurrent phenomenon affecting the cell as a whole, contemporaneous with the drastic changes in the nucleus, was the loss of protein from the cell.

Three to 14 hours following alterations in the protein of the nucleus and cytoplasm, there were noted the first changes in some of the cytoplasmic physiologic processes. The complete breakdown of any one of the 4 major processes of the cell (biosynthesis of enzymes, production of energy, repair and restoration of structural deficiencies, and maintenance of a satisfactory ionic environment) will immedi-

ately result in alterations, usually irreversible, in the other 3 functions. Indeed, when cells were injured with salyrgan, which inhibits the action of many enzymes, there followed in quick succession a loss of energy, a falling apart of the protein structure, and rapid and highly deleterious changes in the ionic constituents of the intracellular fluid. With the destruction of the oxidative and fermentative cycles in the control and irradiated cells, there was a loss of energy available to maintain cellular structure and ionic balance. Protein architectural destruction was increased, or the rate of resynthesis decreased. This inevitably resulted in membrane alterations. A lack of energy to maintain cellular sodium and potassium equilibrium against the normal gradients resulted in a loss of potassium and a gain in sodium. The latter phenomenon was accompanied by a movement of water into the cell.

Denatured protein swells and takes on hydrated sodium ions resulting in further loss of the spatial relationships of the cell architecture. At the very end stage, shortly before the bursting of the cell, trypan blue dye entered both the cytoplasm and the nucleus. At this stage no metabolic activity exists, and the cell is completely disorganized. Examination of cells following rupture of the membrane usually revealed intact cells for many hours. Occasionally a tear in the membrane was seen, but often it was not. After a short initial contraction with release of water and sodium ions, the denatured residual protein structure again became swollen to its initial bursting size, or occasionally a little greater. Gradually over a period of time, protein was dissolved from both the nucleus and the cytoplasm, and the cell became shrunken to an irregular shell of a nucleus with a thin rim of cytoplasm surrounding it. The extravasated protein coagulated into a conglomerate gelatin-like mass.

Some would consider it unrealistic from a biochemical point of view to try to separate the metabolic processes, since the present knowledge concerning their mechanisms shows how intimately different processes are interrelated. For example, coenzyme A appears to be concerned with the metabolism of the 4 major constituents of the cell. A lack of coenzyme A might theoretically result in such widespread and apparently unrelated changes as inability to revise the protein or lipid portion of the plasma membrane or inability to provide the energy for the formation of DNA or the transportation of ions.

Despite the fact that essential key enzymes, coenzymes, vitamins, and hormones may be involved in numerous interrelated metabolic pathways, it nevertheless has been shown in these experiments that the injured cell passes through 4 fairly discrete stages. It cannot be said that all forms of injury require these discrete stages. Cells sub-

jected to large toxic doses of salyrgan passed through the degenerative phases so quickly as to make recognition of each stage almost impossible. It would also appear likely that some toxins and many chemicals would have a lytic effect on the membrane which would result in disorganization of cellular structure and function due solely to ionic imbalance. Competitive analogues may selectively inhibit one stage of protein synthesis without affecting the mitotic process. The discovery and identification of these processes in disease states will constitute the pathology of the future.

In this laboratory we have investigated some of the events leading up to cell death. From the studies described, cellular functions appear to be lost in a reproducible order, regardless of the form of injury. Most of these functions depend on energy production and biosynthesis of enzymes, and these two processes, in turn, are interdependent on each other. Probably the most important and unique property of protoplasm is the ability to duplicate its constituents exactly, and the loss of this quality may be the primary defect when cells fail to recuperate from injury. The delayed end result and sequence of events thenceforth will depend on how much of a given essential metabolite was present before the damage, the amount needed, the natural turnover rate, and the possible alternative paths of synthesis. If this property of self-duplication is that most susceptible to injury, this would explain why mitosis is the process most easily depressed. In no other function of the cell is a more complicated or vital reduplication necessary than in the structure of the genic pattern of the chromosomes. Later, despite the reserve capacities of the cytoplasm, the cell would be unable to duplicate the enzyme pattern essential for the completion of the energy producing reactions. Once energy production is lost, the cell is unable to maintain a normal concentration of ions and water, and the structural organization necessary for life is forever lost.

Summary

Ehrlich tumor cells, when injured by irradiation and a mercurial metabolic inhibitor, pass through 4 discrete, recognizable stages. The fate of the cell is determined by its ability to repair those structures destroyed as well as to maintain synthesis of those molecules normally undergoing a fast turnover in the dynamic equilibrium of the cell.

REFERENCES

1. King, D. W.; Paulson, S. R.; Hannaford, N. C., and Krebs, A. T. Cell death. I. The effect of injury on the proteins and deoxyribonucleic acid of Ehrlich tumor cells. *Am. J. Path.*, 1959, **35**, 369–381.

2. King, D. W.; Paulson, S. R.; Hannaford, N. C., and Krebs, A. T. Cell death. II. The effect of injury on the enzymatic protein of Ehrlich tumor cells. *Am. J. Path.*, 1959, **35**, 575–589.

3. King, D. W.; Paulson, S. R.; Puckett, N. L., and Krebs, A. T. Cell death. III. The effect of injury on water and electrolytes of Ehrlich tumor cells. *Am. J. Path.*, 1959, **35**, 835–849.

4. Lepeschkin, W. W., and Goldman, D. W. Effects of ultrasound on cell structure. *J. Cell. & Comp. Physiol.*, 1952, **40**, 383–397.

5. Rahn, O. Chemistry of death. *Cold Spring Harbor Symposia on Quantitative Biology*, 1934, **2**, 70–77.

6. Lewis, W. H., and McCoy, C. C. The survival of cells after the death of the organism. *Bull. Johns Hopkins Hosp.*, 1922, **33**, 284–296.

7. Evans, H. M., and Schulemann, W. The action of vital stains belonging to the benzidine group. *Science*, 1914, **39**, 443–454.

8. Lewis, M. R. Reversible gelation in living cells. *Bull. Johns Hopkins Hosp.*, 1923, **34**, 373–379.

9. Lewis, M. R. The importance of dextrose in the medium of tissue cultures. *J. Exper. Med.*, 1922, **35**, 317–322.

10. Lewis, W. H. The Relation of the Viscosity Changes of Protoplasm to Ameboid Locomotion and Cell Division. In: A Symposium on the Structure of Protoplasm. Seifriz, W. (ed.). A Monograph of the American Society of Plant Physiologists. Iowa State College Press, Ames, Ia., 1942, pp. 163–197.

11. Moore, R. A. A Textbook of Pathology. Pathologic Anatomy in Relation to the Causes, Pathogenesis and Clinical Manifestations of Disease. W. B. Saunders Co., Philadelphia, 1951, ed. 2, 1048 pp.

12. Lewis, W. H. Observations on cells in tissue-cultures with dark-field illumination. *Anat. Rec.*, 1923, **26**, 15–29.

13. Strangeways, T. S. P., and Canti, R. G. The living cell *in vitro* as shown by dark-ground illumination and the changes induced in such cells by fixing reagents. *Quart J. Micr. Sci.*, 1927–1928, **71**, 1–14.

14. Heinmets, F., and Kathan, R. H. Preliminary studies on the mechanism of biological action of ultraviolet irradiation and metabolic recovery phenomena. Naval Medical Field Research Laboratory (Camp Lejeune, N.C.). Research Project NM 005 052. 27.05, Vol. 5, March, 1954, pp. 29–54.

15. Allfrey, V. G.; Mirsky, A. E., and Stern, H. The chemistry of the cell nucleus. *Advances Enzymol.*, 1955, **16**, 411–500.

16. Gale, E. F., and Folkes, J. P. Effect of nucleic acids on protein synthesis and amino-acid incorporation in disrupted staphylococcal cells. *Nature, London*, 1954, **173**, 1223–1227.

17. Brachet, J., and Chantrenne, H. Protein synthesis in nucleated and non-nucleated halves of *Acetabularia mediterranea* studied with carbon-14 dioxide. (Letter to the editor.) *Nature, London*, 1951, **168**, 950.

18. Malkin, H. M. Synthesis of ribonucleic acid purines and protein in enucleated and nucleated sea urchin eggs. *J. Cell. & Comp. Physiol.*, 1954, **44**, 105–112.

[*Illustrations follow*]

LEGENDS FOR FIGURES

Figures 1 and 2 represent unstained control Ehrlich tumor cells maintained in Krebs-Ringer solution.

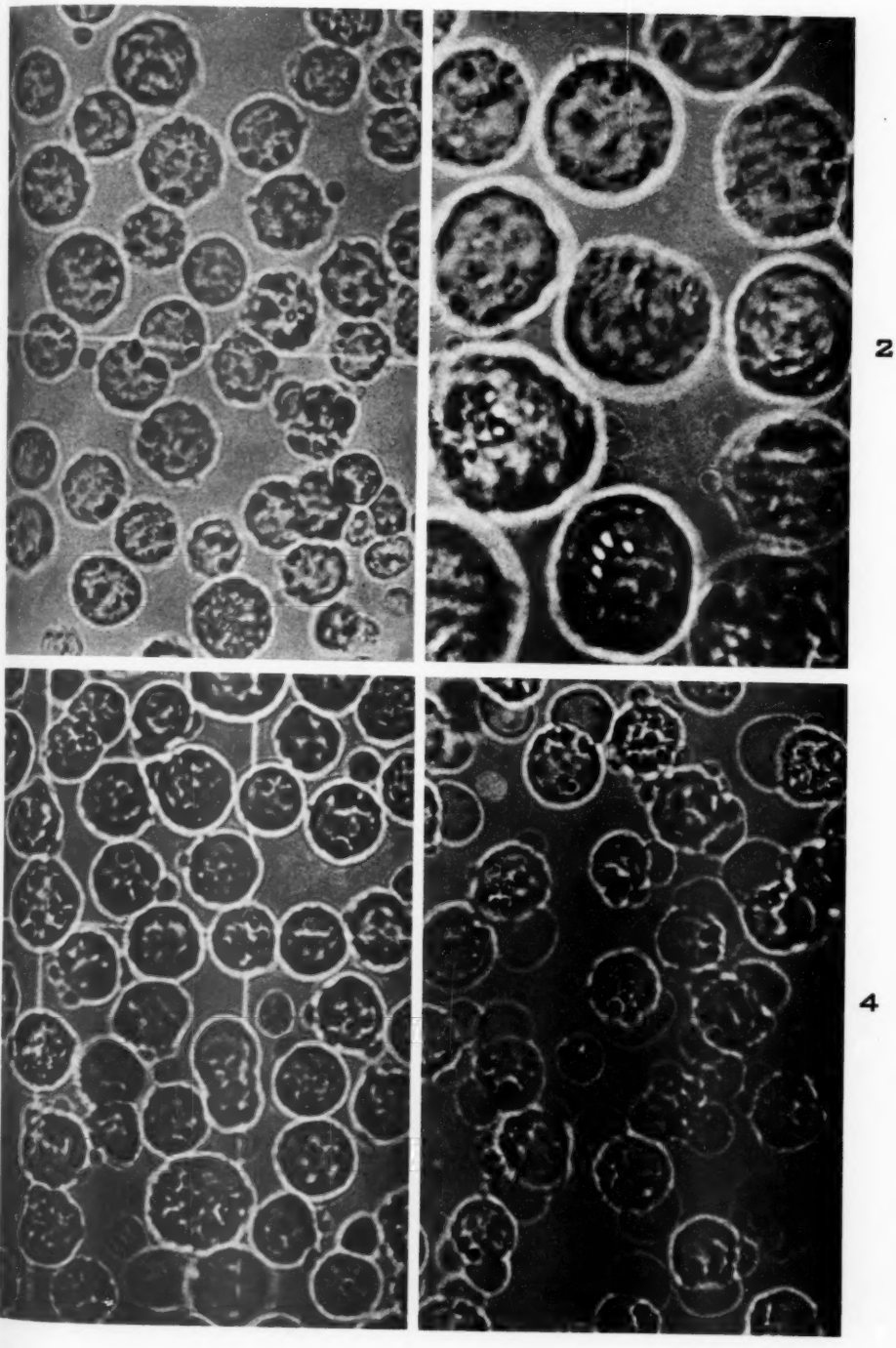
FIG. 1. Control cells immediately after incubation. The cells are spherical, regular in outline and contain numerous refractile granules or vacuoles. Very little cytoplasm is seen, as there is a very high nuclear cytoplasmic ratio in these tumor cells. × 430.

FIG. 2. Control cells 8 hours after incubation. Note the absence of staining with trypan blue. Although they have lost small amounts of protein and DNA and the mitotic index has been reduced, the cells have maintained a normal size, shape and appearance. When .1 cc. of tumor suspension containing 5×10^5 cells was re-injected into mice, 100 per cent of the animals developed tumor. × 970.

Figures 3 and 4 represent irradiated Ehrlich tumor cells maintained in Krebs-Ringer solution.

FIG. 3. Cells immediately after irradiation with 1×10^6 r. The unstained cells are regular in outline and resemble the control cells in every way. × 430.

FIG. 4. One hour following irradiation. The cells have developed irregular cytoplasmic blebs. Some of these appear continuous with the cytoplasm, while others are merely small pockets which do not appear to have protein when stained with hematoxylin and eosin. Although the cells are losing small amounts of DNA and protein, they are maintaining their normal respiration and lactic acid production. There are no marked changes in the electrolyte pattern, and those seen are apparently reversible. × 430.



Figures 5 to 10 represent salyrgan-treated Ehrlich tumor cells.

FIG. 5. Unstained Ehrlich tumor cells immediately after the addition of salyrgan. The cells are small, spherical and regular in outline, resembling the control cells. × 430.

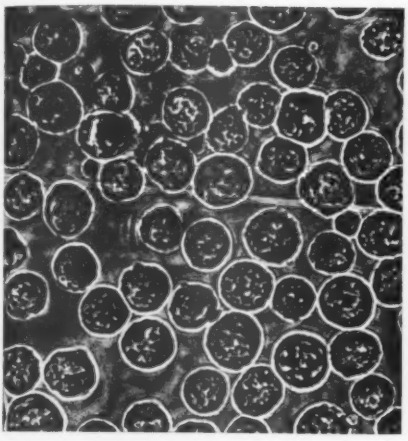
FIG. 6. Fifteen minutes after incubation. The cells have lost large amounts of DNA and protein, but their respiration and lactic acid production have not ceased. The nuclei and cytoplasm are losing potassium, gaining sodium and water, and starting to swell, exhibiting irregular cell membranes and partial clumping of the granules. × 970.

FIG. 7. Forty-five minutes following incubation. Some of the nuclei have stopped swelling and begun to contract. The cytoplasmic protein has a cloudy appearance which probably indicates denaturation or unfolding of the globular proteins. Respiration, lactic acid production and dehydrogenase activity have ceased. × 970.

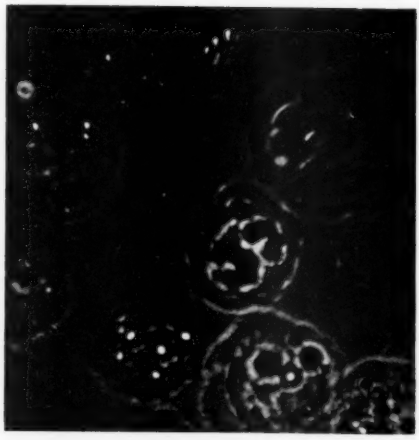
FIG. 8. One hour following incubation. Two cells show rupture of the cell membrane with resultant loss of protein, sodium and water. × 970.

FIG. 9. Two hours following incubation. Following rupture of the cell membrane, the cell maintains an intact structure. Both the nucleus and the cytoplasm stain avidly with trypan blue. For a short time the denatured protein again takes up sodium and water, but this is lost along with the stainability as the protein gradually goes into the surrounding medium. × 430.

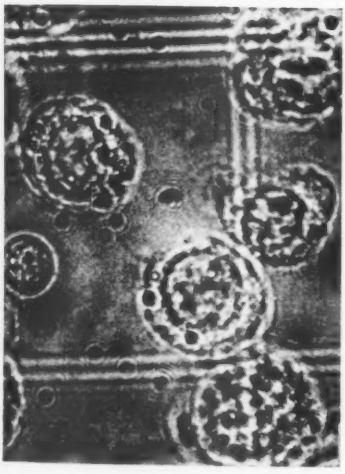
FIG. 10. Two hours following incubation. Note the variation in nuclear size and the presence of a few granules. × 970.



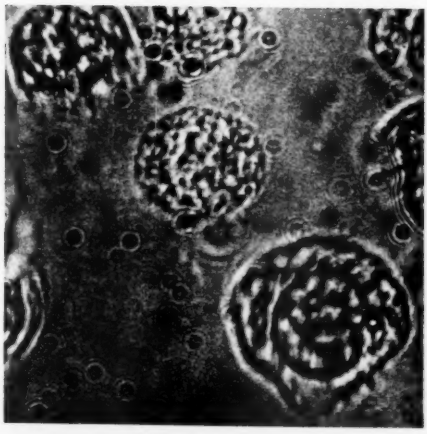
5



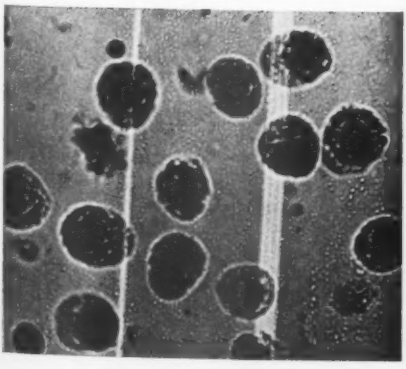
6



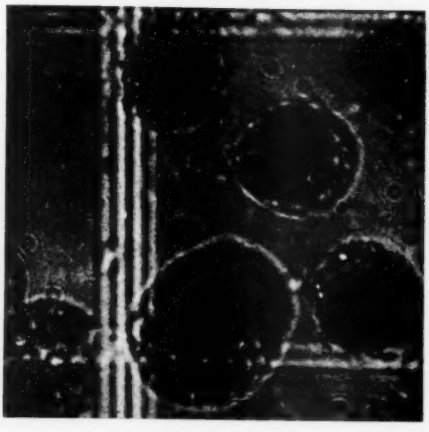
7



8



9



10

THE EXPERIMENTAL PRODUCTION OF ARTERIOSCLEROSIS: RESPONSE OF THE AVIAN ARTERY TO INTRAMURAL CHOLESTEROL AND OTHER INSOLUBLE SUBSTANCES *

MARTIN G. NETSKY, M.D.; THOMAS B. CLARKSON, D.V.M., and DOROTHY STOKES
From the Sections of Neurology, Neuropathology, and Experimental Medicine, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, N.C.

Factors responsible for sclerosis of arteries may be different from those producing the fatty component of atherosclerosis. Plaques large enough to cause inadequate blood flow often contain more fibrous tissue than lipid.[1] The production of the fibrous component in avian atherosclerotic plaques by the injection of cholesterol has been described previously. [2] When a cholesterol suspension was injected into the wall of an avian artery, a foreign body reaction appeared at the site of injection. In addition, a plaque of proliferated collagenous connective tissue was formed beneath the intima. The development of plaques was not affected by adding cholesterol or various fats to the diet.

The present investigation has been undertaken to extend our observations on the response of the avian artery to cholesterol, and to determine whether the intimal proliferative lesions were caused merely by the presence of an insoluble substance or by the chemical action of cholesterol. Paraffin, an insoluble hydrocarbon, was chosen because it has some chemical similarity to cholesterol. Barium sulfate was used because it resembles cholesterol in its insolubility and particulate nature.

METHODS

The technique for intramural injection has been described.[2] White Leghorn cockerels weighing 1 to 1.5 kg. were used. The cholesterol or barium sulfate was injected as a 20 per cent suspension in 0.9 per cent saline solution (0.2 ml.). The paraffin injections consisted of 0.2 ml. of slightly heated fluid paraffin which melted just above the body temperature of the chicken and became solid at 40° C. All injections were made directly into the adventitia or media of the left brachiocephalic artery.

The chickens were maintained on a diet of Purina Growing Mash throughout the experimental periods. Preliminary experiments with

* Supported in part by a grant (B-957) from the Institute of Neurological Diseases and Blindness of the National Institutes of Health, United States Public Health Service.
Presented at the Annual Meeting of the American Association of Neuropathologists, June 14, 1959, Atlantic City, N.J.
Received for publication, March 26, 1959.

cholesterol and saline were carried out with 5 groups of 10 birds each; 2 birds from each group were killed at 2-week intervals to determine the best time for detailed study and the effect of diet.[2] In later investigations, the chickens were divided into 4 groups of 10 birds each. These groups were: saline control, cholesterol injection, paraffin injection, and barium sulfate injection. All birds were killed after 6 weeks, and the right and left brachiocephalic arteries removed for examination. The right brachiocephalic artery served as a control for the spontaneous appearance of arteriosclerosis. The arteries were fixed in 10 per cent neutral formalin.

Frozen sections stained with Sudan IV were prepared from the arteries of 4 birds in each group. The remainder of the arteries were embedded in paraffin and cut in step sections at intervals of 200 μ. The staining techniques used were: hematoxylin and eosin, Sudan IV, periodic acid Schiff (PAS), Verhoeff's elastic tissue stain, phosphotungstic acid hematoxylin (PTAH), and Rinehart's stain for acid mucopolysaccharide.

Results

Most injections were made into the adventitia. A small number were in the media, usually in the outermost portion but never in immediate proximity to the intima.

The intramural presence of cholesterol resulted in two major changes in the artery. A foreign body reaction was seen in the adventitia at the site of injection in every case; in addition, a plaque of connective tissue appeared beneath the intima at a short distance proximal or distal to this site in 75 per cent of the animals. The media itself usually was histologically intact. The foreign body reaction consisted of multinucleated giant cells, cholesterol slits, lymphocytic and mononuclear cells and increased amounts of connective tissue (Fig. 1). Sudan IV stains of the adventitial reaction to cholesterol revealed a large amount of neutral fat (Fig. 2); cholesterol is not sudanophilic. The fat was either free in the tissue spaces or in macrophages; it was not in the foreign body giant cells. In preliminary experiments, the lymphocytic and mononuclear cell response was greatest at 2 weeks, but then decreased. Giant cells and adventitial connective tissue were sparse during the first 2 weeks, but greatly increased thereafter.

The intimal plaques induced by the intramural injection of cholesterol in the adventitia appeared as masses of connective tissue beneath the intima. The commonest response was a small plaque of connective tissue protruding into the lumen (Fig. 3). Fibrin or other blood elements were not seen. The proliferation of connective tissue in some cases was so great that the arterial lumen was almost completely

obliterated (Fig. 4). The plaques gave negative reactions to the PAS and Rinehart stains, and did not contain elastic tissue or fat. The reaction with PTAH was characteristic of collagenous connective tissue. Endothelial cells were occasionally increased in number.

Barium sulfate caused proliferation of connective tissue at the site of injection. However, foreign-body giant cells were lacking, and the inflammatory response was minimal (Fig. 5). Crystals of the injected material were seen in the region of fibrotic reaction. It is noteworthy that the introduction of barium sulfate did not result in the appearance of sudanophilic substance. Only one of 10 birds receiving barium sulfate exhibited an intimal plaque. This plaque gave negative reactions to the PAS and Rinehart stains, did not contain elastic tissue or fat, and was small and tubular rather than focal. The reactive connective tissue here was morphologically different from that encountered in the cholesterol series. The connective tissue cells were concentrically arranged as opposed to a focal disorderly appearance in the reaction to cholesterol. Endothelial cells were not increased in number.

The intramural injection of paraffin elicited a still different reaction in the adventitia and media at the site of injection. There were no foreign-body giant cells. The reactive cells were predominantly lymphocytes accompanied by a few monocytes (Fig. 6). The site of the adventitial reaction contained spaces lined by connective tissue where the paraffin had washed out in the histologic preparation. Local destruction of muscle fibers associated with a lymphocytic response occurred where the paraffin had entered the media. The reaction to paraffin did not contain sudanophilic material. Intimal plaques were not seen.

Spontaneous arteriosclerotic lesions were never found in the control right brachiocephalic arteries.

Discussion

Winternitz, Thomas and LeCompte, in 1938,[3] suggested that cholesterol and its esters, remaining after resorption of hemorrhages from the vasa vasorum, could cause intimal proliferation. Wartman and Laipply[4] did not induce intimal alterations by injecting homologous whole blood into the wall of the femoral artery of dogs. Christianson[5] produced intimal proliferative changes in the dog by the intramural injection of cholesterol dissolved in human fat. Interpretation of these observations is difficult, however, because of the complexity of the injected substances. Leary[6] questioned the hypothesis of Winternitz and his co-workers because "atherosclerosis begins in the most superficial layers of the intima in the region farthest removed from the distribution of vasa vasorum."

The adventitia of arteries does contain numerous vasa. On the basis of our experiments, it is evident that cholesterol in the adventitia is capable of inducing an intimal arteriosclerotic plaque. We have demonstrated that it was not necessary for the cholesterol crystals to be present in the media or subintima to elicit this phenomenon. Furthermore, the intimal plaques induced by intramural injection of cholesterol were frequently not adjacent in the longitudinal plane to the site of adventitial injection. The plaques were located either proximally or distally to the largest part of the adventitial foreign body response to cholesterol.

The large amount of free and intracellular sudanophilic material at the site of cholesterol deposit was of interest because of the frequent association of free fat and cholesterol crystals in human atherosclerotic plaques. Fat was not found, however, within the cholesterol-induced avian plaque. The ability of cholesterol to cause subintimal plaques when injected into the adventitia or media was not shared by barium sulfate or paraffin. The effect of cholesterol, therefore, was characteristic of this substance and was not merely the response of the artery to an insoluble or foreign compound. Fat was not seen at the sites of injection with either barium sulfate or paraffin.

The 3 coats of the arterial wall differed greatly in their biologic responses to the 3 injected substances. The adventitia had the most diverse reactions. Cholesterol caused a foreign-body reaction and a transient inflammatory reaction, proliferation of connective tissue and free fat; barium sulfate caused only an increase in connective tissue; whereas paraffin resulted in an inflammatory reaction, predominantly of lymphocytes.

By contrast, the media had little reactivity. Increased amounts of connective tissue rarely occurred as a result of the injections, and when present, appeared as extensions from the adventitia. Usually the media suffered a focal loss or interruption of elastic fibers, or was normal in appearance.

The intima had an even more stereotyped reaction. Plaques of connective tissue usually formed after the intramural introduction of cholesterol, and rarely or not at all after the introduction of barium sulfate. In the case of paraffin, the intima was not altered at all. Injury to the intima by other means results in formation of increased amounts of connective tissue. This has been shown by Duguid[7] who passed threads through the arterial wall of dogs, and by Christianson,[4] using intramural cholesterol dissolved in human fat.

SUMMARY

The injection of cholesterol into the adventitia of the avian artery caused a reaction consisting of foreign-body giant cells, cholesterol slits, and increased amounts of connective tissue. Free fat was abundant in the tissue spaces and within macrophages at the site of injection. Also induced was a focal proliferation of connective tissue in the intima in 75 per cent of the birds, even though the media was histologically intact. The plaques ranged in size from small elevations of the intima to almost complete occlusion of the arterial lumen.

The intramural injection of paraffin or barium sulfate induced a response at the site of injection which differed from that caused by cholesterol. The response was largely of lymphocytes when paraffin was injected, and of connective tissue with barium sulfate. Neither of these substances was accompanied by an accumulation of sudanophilic material. A small intimal plaque was seen in only one of 10 birds receiving the barium, and none occurred after paraffin injection. The response of the avian artery to intramural cholesterol, then, had some specificity, because plaque formation did not occur with the other compounds tested.

The 3 layers of the avian arterial wall differed greatly in their reaction to foreign substances inserted intramurally. The adventitia had diverse reactions; the media suffered only focal loss of elastic fibers. The intima appeared to be capable of only one reaction, the formation of a plaque of connective tissue, when the appropriate stimulus was delivered.

REFERENCES

1. Paterson, J. C. The reaction of the arterial wall to intramural hemorrhage. In: Symposium on Atherosclerosis. Publication 338, National Academy of Sciences. National Research Council, Washington, D.C., 1954, pp. 65–73.
2. Netsky, M. G., and Clarkson, T. B. Response of arterial wall to intramural cholesterol. *Proc. Soc. Exper. Biol. & Med.*, 1958, **98**, 773–774.
3. Winternitz, M. C.; Thomas, T. M., and LeCompte, R. M. The Biology of Arteriosclerosis. Charles C Thomas, Springfield, Ill., 1938, 142 pp.
4. Wartman, W. B., and Laipply, T. C. The fate of blood injected into the arterial wall. *Am. J. Path.*, 1949, **25**, 383–388.
5. Christianson, O. O. Observations on lesions produced in arteries of dogs by injection of lipids. Lipids injected: human fat, fatty acids, soaps, and cholesterol. *Arch. Path.*, 1939, **27**, 1011–1020.
6. Leary, T. Vascularization of atherosclerotic lesions. *Am. Heart J.*, 1938, **16**, 549–554.
7. Duguid, J. B. Diet and coronary disease. *Lancet*, 1954, **1**, 891–895.

[*Illustrations follow*]

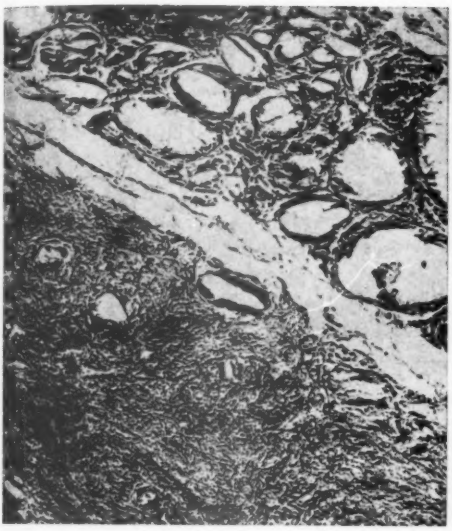
LEGENDS FOR FIGURES

FIG. 1. An adventitial reaction to cholesterol, showing proliferation of connective tissue. The spaces in the upper part of the field are cholesterol slits surrounded by multinucleated giant cells. Hematoxylin and eosin stain. × 120.

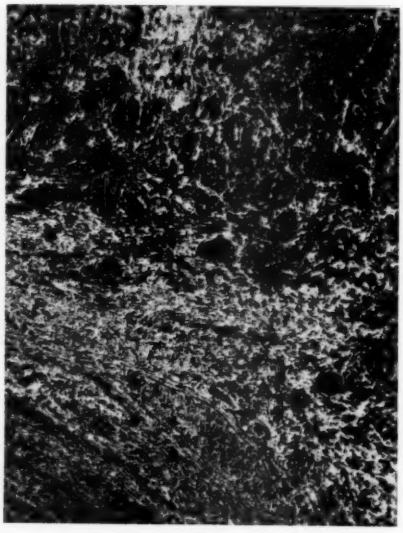
FIG. 2. An adventitial reaction to cholesterol. Masses of lipid appear in the tissue spaces and within macrophages. The lower part of the field contains a few muscle fibers within a fibrotic reaction. Sudan IV stain. × 90.

FIG. 3. A small plaque (arrow) beneath the intima appearing in response to cholesterol injection. A typical adventitial reaction is present. Note the intact media. Hematoxylin and eosin stain. × 110.

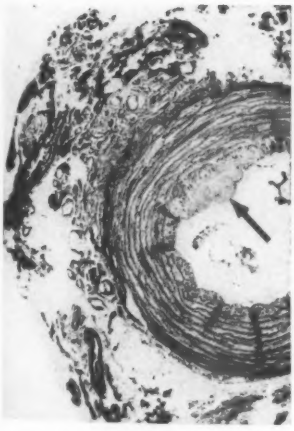
FIG. 4. Reaction to cholesterol. The adventitial response is massive. A subintimal plaque almost occludes the lumen. This is not thought to be an organized thrombus. Hematoxylin and eosin stain. × 20.



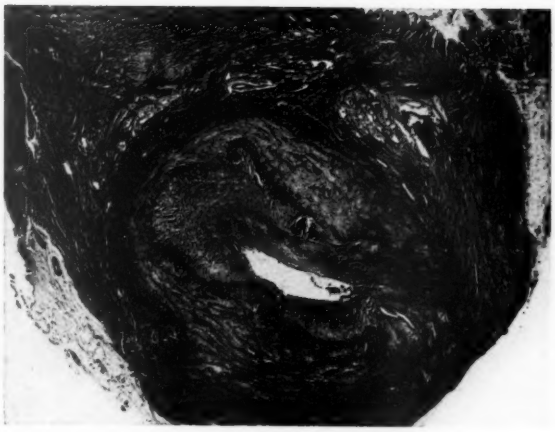
1



2



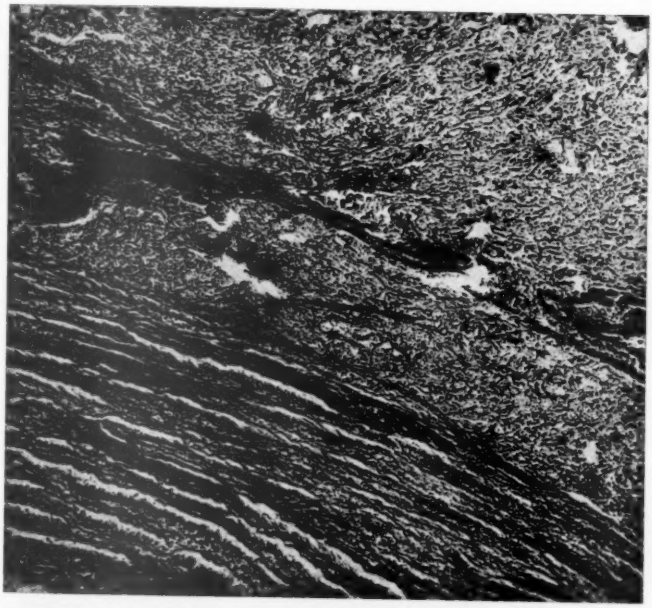
3



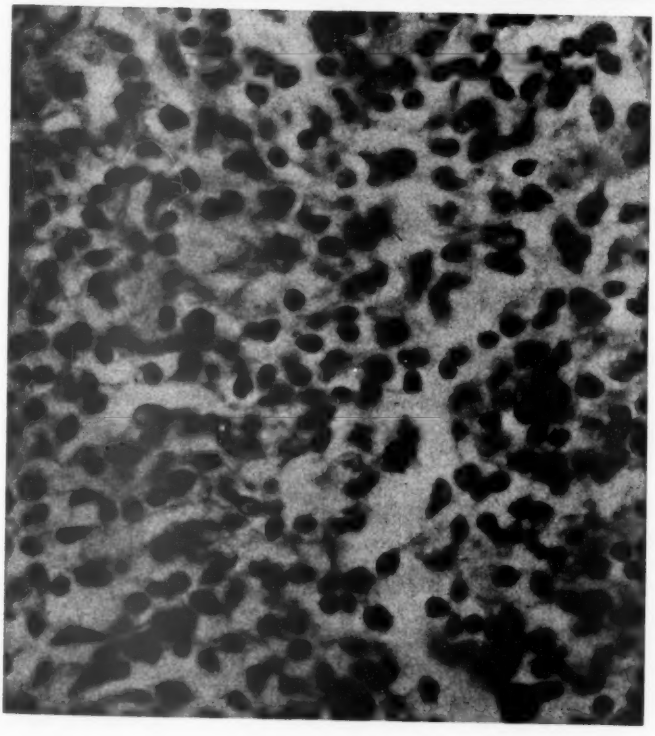
4

FIG. 5. Adventitial reaction to barium sulfate. Moderately dense connective tissue is present in the upper part of the field. A few muscle fibers have been elevated, but the media is otherwise intact. Crystals are present but are not shown in the photograph. Hematoxylin and eosin stain. × 120.

FIG. 6. Adventitial reaction to paraffin. The predominant cells are lymphocytes. Hematoxylin and eosin stain. × 480.



5



6

Notice to Subscribers

The American Journal of Pathology will be issued monthly (two volumes per year) beginning with Volume 36, January, 1960.

Publication and circulation will be handled by Paul B. Hoeber, Inc., Medical Book Department of Harper & Brothers, 49 East 33rd Street, New York 16, N.Y.

Subscriptions effective after December 31, 1959, should be sent to the above address, payable to Paul B. Hoeber, Inc. There will be no increase in subscription rates ($15.00 annually, plus $.50 postage to Canada and $1.00 postage to all other countries).

Manuscripts should be submitted to the Editorial Office which will continue at its present location: *The American Journal of Pathology,* Cincinnati General Hospital, Cincinnati 29, Ohio.

—Edward A. Gall, M.D., *Editor-in-Chief*

Announcement

Fifty-seventh Annual Meeting

of the

American Association of Pathologists and Bacteriologists

to be held

April 28, 29 and 30, 1960

at the

Hotel Peabody, Memphis, Tennessee

Symposium

Friday, April 29, 1960

"Genetic Factors in Disease"

Referee, Dr. Nash Herndon

Titles for presentation at the regular sessions and the Symposium should be submitted to the Secretary of the Association **not later than January 15, 1960.** Abstracts will not be published in *The American Journal of Pathology* as in the past. It is expected that there will be pre-publication of abstracts in the Program to be sent out approximately one month in advance of the meeting.

Secretary: Russell L. Holman, M.D.
Louisiana State University
School of Medicine
New Orleans 12, Louisiana